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SECTION MEETINGS

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Human Infection with Rift Valley Fever Virus and Immunity Twelve Years After Single Attack.*

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The virus of Rift Valley Fever (a disease of sheep and cattle in Kenya Colony, British East Africa) was first isolated in 1930 by Daubney, Hudson and Garnham,[1] who also established its pathogenicity for human beings. Since that time there have been accidental human infections in practically every laboratory in which this virus has been studied—4 cases in Kenya[1] in 1930, 4 cases in 1931 in London[2] and another in 1934,[3] in New York 1 case in 1932,[4] 3 cases in 1933[5] and 3 cases in 1934,[6] and at least 1 case in Uganda[7] in 1944—a total of 17. The purpose of the present communication is not merely to record still another instance of accidental laboratory infection, but rather to indicate (a) that a strain of virus which has had at least about 300 brain to brain passages in mice has not undergone any modification in the type of disease it can produce in human beings, and (b) that neutralizing antibodies for the virus can persist for at least 12 years after a single attack of

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* This investigation was conducted with the aid of the Commission on Virus and Rickettsial Diseases, Army Epidemiological Board, Office of the Surgeon General, U. S. Army, Washington, D.C.

[1] Daubney, R., Hudson, J. R., and Garnham, P. C., *J. Path. and Bact.*, 1931, **34**, 545.

[2] Findlay, G. M., *Tr. Roy. Soc. Trop. Med. and Hyg.*, 1932, **25**, 229.

[3] Findlay, G. M., quoted by Kitchen.[5]

[4] Schwentker, F. F., and Rivers, T. M., *J. Exp. Med.*, 1934, **59**, 305.

[5] Kitchen, S. F., *Am. J. Trop. Med.*, 1934, **14**, 547.

[6] Francis, T., Jr., and Magill, T. P., *J. Exp. Med.*, 1935, **62**, 433.

[7] Smithburn, K. C., personal communication concerning case at Yellow Fever Research Institute, Entebbe, Uganda, East Africa.

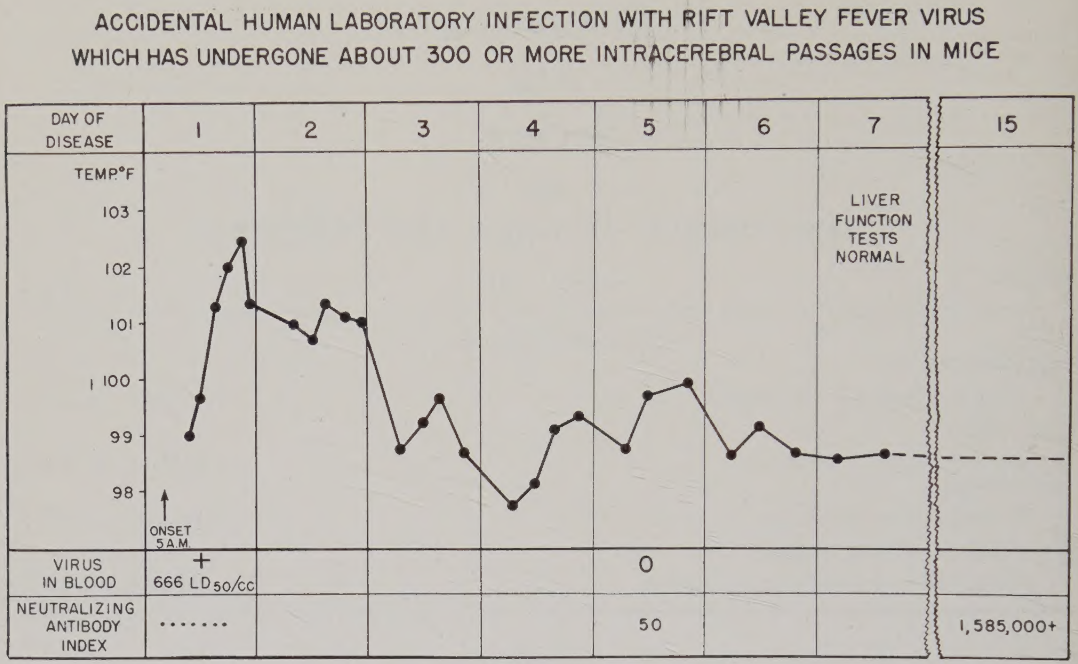

FIG. 1.

the disease without further exposure to the virus.

The "K" strain of virus was obtained from Dr. N. Ishii of Japan in 1945 and was alleged to be a strain of mouse-adapted dengue virus. However, studies, which will be reported at a later date, established that the "K" virus was a strain of Rift Valley Fever virus. The history of the virus prior to the time it came to be called "dengue" is not known, although it is known that Rift Valley Fever virus was brought into Japan from France for laboratory investigations. It was stated that the "K" virus had undergone 293 brain to brain passages in mice since 1942 and it had had 5 additional intracerebral passages in mice in Cincinnati, when the accidental human infection to be reported here had occurred. One of us (A.B.S.) worked continuously with this virus from October 26, 1945 until early January, 1946, without becoming infected or developing neutralizing antibodies for the virus. He resumed work with this virus on October 31, 1946 when he ground a number of infected mouse brains in a mortar and performed an intra-abdominal neutralization test in mice, at which time contamination of the skin or aspiration of droplets of virus might have occurred. Between October 31 and the onset of illness on November 6, the only other activity related to this virus consisted of the daily examination of the inoculated mice.

A.B.S., who had been in good health, was awakened from his sleep at 5 a. m. on November 6 by a very severe frontal headache. Acetylsalicylic acid and codeine were taken at that time and since they brought no relief, no more was taken during the course of the illness so that the temperature curve (rectal temperatures shown in Fig. 1) was not subsequently affected by antipyretics. The temperature did not rise until the late afternoon and evening. Severe aching in the muscles, bones, and joints, and anorexia were the only other symptoms which developed during the course of the day and persisted for about 48 hours. The temperature returned to normal on the 3rd day and only mild muscle aching and weakness remained. He was out of bed on the 4th day and back in the laboratory on the 5th day. The low-grade fever up to 99.9° on the 5th day was associated with a feeling of feverishness and slight malaise, but recovery was complete and uneventful thereafter. It may be of interest

TABLE I.
Immunological Identity of Virus Recovered from Patient "A.B.S." and "K" Strain of Rift Valley Fever Virus.

Intra-abdominal Neutralization Test in Mice.

Dilution of virus in mixture	Mortality in mixture with: Normal rabbit serum	"K" hyperimmune rabbit serum
10^{-1}	—	0, 0, 0, 0
10^{-2}	1+*, 1+, 1+, 1+	0, 0, 0, 0
10^{-3}	1+, 1+, 1+, 1+	0, 0, 0, 0
10^{-4}	2, 2, 2, 3	0, 0, 0, 0
10^{-5}	2, 2, 2, 2	0, 0, 0
10^{-6}	2, 2, 0, 0	0, 0, 0, 0
10^{-7}	3, 0, 0, 0	

* Each digit represents day of death of each mouse used in the test; 1+ = more than 24 but less than 36 hours; 0 = survival.

to note that the following tests, for which we are indebted to Dr. S. Rapoport, carried out on the blood 6 days after onset revealed no abnormality in liver function: Total bilirubin—0.3 mg %; cephalin flocculation negative; phosphorus—3.2 mg % and phosphatase—3.2 Bodansky units; total protein—7.6 g % with an albumin-globulin ratio of 2.8.

Approximately 10 hours after the first appearance of headache, blood was drawn and inoculated in mice—0.5 cc of defibrinated blood intra-abdominally into each of 5 mice and 0.03 cc of the serum intracerebrally into each of 5 additional mice. All of these mice succumbed within 48 hours, and 3 successful serial passages by the intra-abdominal or intracerebral routes were carried out. Extensive hepatic necrosis and acidophilic intranuclear inclusions were present in the liver cells of the first passage mice. The immunological identity of the virus recovered from the blood of A.B.S. and the "K" strain of Rift Valley Fever virus was established by the results of the intra-abdominal neutralization test shown in Table I. Varying dilutions of mouse brain suspension containing the 3rd passage of the "A.B.S." virus were mixed with equal parts of "K" hyperimmune rabbit serum and for control a similar set was prepared with normal rabbit serum. After incubation of the mixtures in a water bath at 37°C for about one hour, 0.1 cc was injected intra-abdominally into approximately 4-week-old Swiss mice. Some of this

TABLE II.
Early Development and Long Persistence of Neutralizing Antibodies for Rift Valley Fever Virus ("K" Strain) After a Single Attack of the Disease in Human Beings.

Neutralization Tests by Intra-abdominal Route in Mice.

Test	Serum	Interval between onset and specimen	Mortality at indicated dilutions of virus in mixtures 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	LD_{50}	Neutralization index
A	Normal rabbit control	—	—	—	—	2,2,3,3†	2,2,2,2	2,2,2,5	6,0,0,0	$10^{-6.7}$	—
	Patient A.B.S.—1946	4 days	2,2,2,2	2,2,2,2	2,2,2,2	2,2,2,2	2,3,0,0	0,0,0,0	—	$10^{-5.0}$	50
	Patient A.B.S.—1946	14 "	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	—	$10^{-0.5}$—?	1,585,000+?
	R.W.B.—1946	Specimen 1	2,2,2,2	2,2,2,2	2,2,2,2	2,2,2,2	2,2,2,2	2,3,3,5	—	$10^{-6.5}$+?	2 — ?
	worked with virus	Specimen 2, 13 days later	2,2,2,2	2,2,2,2	2,2,2,2	2,2,2,2	2,2,2,0	2,2,0,0	—	$10^{-5.8}$+?	8 — ?
B	Normal rabbit control	—	—	—	2,2,2,2	2,2,2,4	2,2,2,2	2,2,2,2	0,0,0,0	$10^{-6.5}$	—
	Patient K.C.S.—1944	16 mo	—	—	5,8*,9,0	7,0,0,0	0,0,0,0	0,0,0,0	—	$10^{-3.5}$	1,000
	Patient T.F.—1934	12 yrs	6,8‡,10,0	4,6,6,0	8*,10,0,0	7,10,0,0	0,0,0,0	0,0,0,0	—	$10^{-3.0}$	3,160

† Each digit represents day of death of each mouse used in the tests. 0 = survival.

* Rift Valley Fever virus shown to be cause of death by passage of liver.

‡ No virus recovered by passage of liver.

same preparation of "K" hyperimmune rabbit serum was sent to Dr. Kenneth Smithburn at the Yellow Fever Research Institute in East Africa who reported that it neutralized their viscerotropic Rift Valley Fever virus.

In order to determine how much virus was present in the blood at the beginning of the human infection, some of the serum obtained 10 hours after onset and kept frozen in a CO_2 box was titrated by intracerebral inoculation of mice. It was rather surprising to find only 666 LD_{50} of virus per cc of serum (the titer was $10^{-1.3}$ for 0.03 cc), in view of the demonstration by Kitchen[5] that the blood of sick mice contained at least 100 billion LD_{50} of virus per cc. Another specimen of the patient's serum taken on the 5th day, coincident with the slight elevation in temperature, contained no virus but a definite, though small, amount of neutralizing antibody was already present (Table II).

R.W.B. first began working with the "K" virus on October 30, 1946 and because he felt feverish (temperature not taken) and had a moderately severe headache on the night of November 5, his blood taken on November 6 was inoculated in mice in the same manner and at the same time as that of A.B.S. However, no virus was recovered. The results of the neutralization tests against the "K" virus with the sera obtained from both A.B.S. and R.W.B., shown in Table II, indicate that only A.B.S. developed neutralizing antibodies and that the titer in his case had risen from 50 in the serum taken 4 days after onset to at least 1,585,000 in the serum taken 10 days later. The results of test B (Table II) are of especial interest because they suggest that after an initial high peak, the neutralizing antibody drops to a titer of about 1,000 and can then persist for at least 12 years without any further exposure to the virus in the interim. Both K.C.S.[†] and T.F.[‡] suffered from accidental laboratory infections, and T.F. has had no further exposure to Rift Valley Fever virus since he stopped working with it in 1934.

Discussion. The clinical features and the duration of the febrile phase of the illness reported in this communication are no different from the human cases which resulted from exposure to lamb or early mouse-passage virus. Kitchen[5] quotes a personal communication from G. M. Findlay regarding a human laboratory infection with virus which had undergone more than 400 successive passages in mice, the route and tissue used for passage not being indicated however; this case probably had a more protracted course than usual since it is stated that the virus was recovered from the blood on the 6th day of illness. The absence of any appreciable modification in the virulence of this virus for human beings by approximately 300 or more intracerebral passages in mice is to be contrasted with the rapid modification which occurs when dengue virus is passaged by this route in mice.[8] The chances for modification or mutation would appear to be much less for a virus, such as that of Rift Valley Fever, which, without requiring any preliminary adaptation, multiplies readily in mice, than for a virus which, like that of dengue, can be made to propagate in mice only with the greatest difficulty. The disease produced by the virus of Rift Valley Fever has previously been compared to dengue,[1] sandfly fever[1] and influenza.[4,5] However, one of us (A.B.S.) having spent several years studying both dengue and sandfly (pappataci) fever, finds that human infection with Rift Valley Fever virus is clinically indistinguishable from sandfly fever, but unlike the type of disease produced in the majority of human beings as a result of primary infection with various types of dengue virus. It has indeed been suggested[1] that what has in the past been diagnosed as 3-day fever or sandfly fever in certain parts of East Africa may very well be examples of Rift Valley Fever in man. Fortunately the differentiation is easy in the laboratory since the blood of patients, with sandfly fever or dengue, inoculated intra-abdominally in mice, is without effect, while in the case of Rift Valley Fever the mice die in about 2 to 3 days.

[†] We are indebted to Dr. J. E. Smadel of the Army Medical School for this specimen of serum and to Dr. Kenneth C. Smithburn of the Rockefeller Foundation for the details of the case.

[‡] We are indebted to Dr. Thomas Francis, Jr., of the University of Michigan for the serum on this case (described in reference 6).

[8] Sabin, A. B., and Schlesinger, R. W., *Science*, 1945, **101**, 640, and unpublished observations.

Findlay[9] has previously reported the persistence of neutralizing antibodies for the virus of Rift Valley Fever for 4 to 5 years in laboratory workers, who, however, might have had further exposures to the virus. The present demonstration of the persistence of the antibodies for a period of 12 years, is an unusual example of the duration of immunity after a single attack without further exposure to the virus during the intervening years.

Summary. 1. Accidental human laboratory infection with a strain of Rift Valley Fever virus which had undergone at least about 300 intracerebral passages in mice, indicated that no modification in its pathogenicity for man resulted from this mode of passage.

2. The virus was recovered from the blood 10 hours after the first appearance of headache but titration revealed that the virus content was very low—only 666 LD_{50} per cc of serum. Four days after onset, no virus was detected in the blood, but neutralizing antibodies (index of 50) were already present and in the next 10 days increased to yield an index of at least 1,585,000.

3. Neutralizing antibodies for Rift Valley Fever virus (index of 3,160) were found 12 years after a single attack in the serum of an individual who had no further exposure to the virus during the intervening years.

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[9] Findlay, G. M., *Brit. J. Exp. Path.*, 1936, **17**, 89.

15804 P

Streptomycin in the Therapy of Granuloma Inguinale.*

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(Introduced by G. L. Kelly.)

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The use of antimonial preparations in the therapy of granuloma inguinale is well established. Its limitations, particularly in chronic cases, have long been known. Recurrences of the disease have occurred with a great degree of frequency even after apparent healing was attained. Other forms of treatment have been employed alone or in conjunction with the antimonials and have ranged from the local application of escharotic agents to surgery and x-radiation therapy. Although cure may be attained by adhering to one or another of these procedures, much is to be desired in the management of this minor venereal disease.

With the advent of the antibiotics it was hoped that a more adequate and specific therapy for granuloma inguinale might be evolved. Preliminary observation with penicillin and tyrothricin showed that these drugs were of little value. One to 4 million units of penicillin administered for the expressed purpose of treating granuloma inguinale failed to control the granulomatous lesions. Tyrothricin was also without effect when it was applied locally in the treatment of granuloma inguinale.

Streptomycin, however, has proved so strikingly effective in the therapy of granuloma inguinale that a preliminary report is warranted at this time. A total of 23 patients has received streptomycin for granuloma inguinale. The diagnosis of the disease was established in all cases by demonstrating the presence of Donovan bodies in either spreads made from the lesion or by

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* This study was aided by a grant from the United States Public Health Service.

histologic study of biopsy sections.[†] It is to be emphasized that no other form of treatment was instigated. The daily doses employed ranged from 0.3 to 1 g administered in 6 equal doses per 24 hours at 4-hour intervals. Total doses varied from 3.3 to 46 g given over a period of time extending from 6 to 46 days. The patients to whom the drug was administered may be divided into 3 groups: (1) untreated patients, (2) chronic cases inadequately treated over a period of years, (3) patients who had relapsed after an extensive course of fuadin or anthiomaline therapy.

Daily smears made from lesions after streptomycin injections showed that Donovan bodies could not be demonstrated 5 to 9 days after administration of the antibiotic. Fusospirochetosis, often observed as a superimposed infection, was controlled with but few exceptions within 48 to 72 hours. Healing followed a general pattern and was usually centripetal in effect. The extragenital lesions responded most rapidly to treatment. On the other hand, when moist, ulcerated areas were in constant contact with one another, as between the thigh and scrotum, response to therapy was slower. However, the Donovan bodies disappeared as rapidly in these recalcitrant lesions as in those that showed rapid clinical improvement. The lesions ultimately were covered by depigmented scar tissue.

Toxic reactions were noted in 2 patients. One occurred in a colored male 29 years of age after 10 days of administration of a total dose of 6 g of streptomycin. The toxic manifestations were expressed as a febrile reaction associated with a maculo-papular rash of the extremities and malar regions of the face. In addition a fine vesicular eruption and edema of the lips were also observed. Pruritus accompanied the skin eruption. Fifty mg of benadryl administered twice a day failed to control or prevent the reaction from occurring when streptomycin was tried again. Another patient developed a mild burning of the conjunctiva also after 10 days of treatment with a total dose of 3 g of streptomycin. However, after discontinuing the drug for 4 days, resumption of the same daily dose failed to cause any untoward symptoms.

Follow-up examinations, while meager at present, indicate that relapses may occur. Two patients who had received less than 4 g of the drug returned with a recrudescence of the lesion. A third patient who had received 28 g suffered a relapse several months later. Donovan bodies were readily demonstrable in the recurrent lesions of these three cases. More time must elapse before a true evaluation of the overall effectiveness of streptomycin can be established. Certainly the remarkable clinical improvement and the rapid disappearance of the Donovan bodies from the smears of patients are reassuring and indicate that streptomycin is the most effective agent available today for the treatment of granuloma inguinale.

[†] A note of appreciation is due Dr. Edgar R. Pund for the examination of these tissue sections.

15805

Streptomycin in Treatment of Experimental Trypanosomiasis in White Mice and Chick Embryos.*

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The striking results obtained by the use of streptomycin and penicillin, in certain bacterial diseases, has somewhat overshadowed their use against fungus and protozoan infections. With respect to trypanosomiasis, Neghme,[1] working with *T. cruzi* in mice, and Augustine, *et al.*,[2] investigating *T. lewisi* in rats, have reported completely negative results in the treatment of experimental trypanosomiasis with penicillin. More recently Schatz and colleagues[3] have described an attempt to isolate an antibiotic active against *T. equiperidum.* In their work they found that a crude preparation of streptomycin immobilized the trypanosomes in freshly drawn rat blood but only when used in a concentration greater than 1:200. Anderson, Villela and Reed[4] were unable to show cytolysis of *T. equiperidum* by penicillin or streptomycin; and subtilin, in their hands, failed to prolong the life of mice infected with this organism. The use of streptomycin in human trypanosomiasis, particularly Chagas disease, has been somewhat limited but from information available,[5] the results have been regularly disappointing.

The following report presents the results of a study on the influence of streptomycin in experimental infections of mice and chick embryos with *T. brucei, T. equiperidum* and *T. hippicum.*† These flagellates are maintained in the laboratory stock culture collection by serial passage in guinea pigs. In the present study the blood of infected rats was used as the source of organisms.

Experimental Procedure. White Mice. White mice were inoculated subcutaneously with blood taken from the tail of an infected rat. Streptomycin was administered subcutaneously at 3-hour intervals, for a period of 4 days, beginning 24-36 hours after the preliminary inoculation. Total dosage ranged up to 16,000 units per mouse. Addinall[6] gives the LD_{50}‡ for mice as 750 mg per kg when given subcutaneously. Two control series were included in each experiment; (1) the trend of the infection in untreated mice and (2) the toxicity of the antibiotic for normal mice. The course of the disease was followed by making microscopical examinations of blood specimens taken, by snipping the tip of the tail, at 12- to 24-hour intervals. These examinations were made with the aid of dark-field illumination and the number of trypanosomes per oil immersion field (diameter 145 μ) was determined and recorded.

Chick Embyros. Fertile eggs, which had been incubated 10 days, were inoculated with heart's blood from an infected rat. The material was injected directly into the yolk sac using a 1½-inch, 20-gauge needle and entering from the air sac end of the egg. This

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* Streptomycin, as the hydrochloride, was made available for this study through the courtesy of Merck and Co. and Abbott Laboratories.

[1] Neghme, Amador, *Science*, 1945, **101**, 115.

[2] Augustine, D. L., Weinman, D., and McAllister, J., *Science*, 1944, **99**, 19.

[3] Schatz, A., Magnuson, H. J., Waksman, S. A., and Eagle, H., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 143.

[4] Anderson, H. H., Villela, G. G., and Reed, R. K., *Science*, 1946, **103**, 419.

[5] Personal communication of Dr. M. H. Soule.

† The original sources of the trypanosome strains used in this study were: (1) *T. brucei*—lineally descended from the original isolation of Bruce (1896). Obtained from Dr. Thomas, Liverpool School of Tropical Medicine, about 1900. (2) *T. equiperidum*—from Dr. A. L. Tatum, University of Wisconsin. (3) *T. hippicum*—from Dr. Herbert Clark, Gorgas Memorial Institute, Ancon, Canal Zone.

[6] Addinall, C. R., *The Merck Report*, October, 1945.

‡ Lethal dose for 50% of mice treated.

TABLE I.
The Effect of Stroptomycin on Various Trypanosome Infections in White Mice.

Days after inoculation	2	3	4	5	6	7	8	9
T. brucei + streptomycin	0/s*	1/f†	15/f	AC‡	\§	Dead		
’’ ’’ control	0/s	1/s	1/5f	10/f	\	’’		
T. equiperidum + streptomycin	1/s	1/10f	3/f	AC	AC	’’		
’’ ’’ control	1/s	1/10f	2/f	15/f	AC	’’		
T. hippicum + streptomycin	0/s	0/s	1/s	\	1/10f	10/f	25/f	Dead
’’ ’’ control	0/s	0/s	1/s	\	10/f	AC	AC	’’

* 0/s = no trypanosomes present on slide.
† 1/f = 1 trypanosome per oil immersion field.
‡ AC = too numerous to count.
§ \ = no count made.

TABLE II.
Effect of Streptomycin on Various Trypanosome Infections in Chick Embryos.*

Days after inoculation	3	4	5	6	7	8	9
T. brucei + streptomycin	0/s	0/s	\	1/10f	5/f	Dead	
’’ ’’ control	0/s	0/s	\	2/f	5/f	’’	
T. equiperidum + streptomycin	0/s	1/s	2/f	3/f	15/f	\	Dead
’’ ’’ control	0/s	1/10f	5/f	10/f	15/f	\	’’
T. hippicum + streptomycin	0/s	\	0/s	\	1/s	1/f	’’
’’ ’’ control	0/s	\	0/s	\	1/10f	1/3f	’’

* See footnotes to Table I.

hole was immediately sealed with sterile paraffin. The streptomycin was introduced through the same opening as the original inoculation after thorough application of tincture of iodine to the paraffin seal. Three treatments were given every 24 hours for a period of 4 days. The hole was resealed after each injection of streptomycin. The total dosage in several instances was 40,000 units. The same controls, *i.e.* normal infection and toxicity, were used as were included in the mouse series.

Samples of blood, for microscopic examination, were obtained by nicking the shell of the egg with a carborundum disc previously treated with tincture of iodine.[7] The openings were made in a region rich in capillaries but not overlying the large vessels. The orifice was, in each case, carefully sealed with sterile paraffin. Uninfected embryos were subjected to the same treatment, sterile saline solution being substituted for the streptomycin. These embryos served to indicate damage due to trauma. The microscopic studies were made with the aid of dark-field illumination and the results recorded as previously described.

Results. Typical examples of the findings, with the infection in mice, are presented in Table I. Each of the animals received 600 units of streptomycin at 8:30 a. m., 11:30 a. m., 2:30 p. m. and 5:30 p. m. and 1,600 units at 8:30 p. m. for a period of 4 days, making a total of 16,000 units for each mouse.

As shown by Table I, there was no significant difference in either the course or the duration of the trypanosome infection in treated and untreated mice. In certain other experiments, using *T. brucei,* a total quantity of 28,000 units of streptomycin per mouse was administered with the same results. The toxicity controls showed no ill effects from any of the quantities used.

Table II gives a typical example of the results of the experiments with chick embryos. Each embryo was treated with 2,000 units of streptomycin at 10:30 a. m. and 2:30 p. m. and 6,000 units at 5:30 p. m. for a period of 4 days, making a total of 40,000 units for each embryo.

The data in Table II clearly indicate that there was no significant difference in the course or duration of the infection between treated and untreated embryos. The toxicity controls survived and many hatched.

The data recorded in this series of experiments confirm the findings of Hood,[7] who made a thorough investigation of the course of infection of trypanosomiasis in animals and chick embryos with the same strains of organisms used in this study.

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[7] Hood, Mary N., unpublished thesis.

Summary. White mice and chick embryos were infected with *T. brucei*, *T. equiperidum* and *T. hippicum*. After a lapse of 24-36 hours streptomycin was administered subcutaneously to the mice and was injected into the yolk sac of the chick embryos. Each mouse received a total of 16,000 units and each chick embryo, a total of 40,000 units of streptomycin. The course of the disease was followed by microscopic examination of blood specimens taken at regular intervals. As far as could be determined, this antibiotic agent did not alter the course of the infections or prolong the life of the treated mice or embryos.

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Low Toxicity of Sulfonamide Mixtures. II. Combinations of Sulfathiazole, Sulfadiazine and Sulfamerazine.*

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A new and simple approach to the prevention of renal complications caused by sulfonamides was presented in a previous communication.[1] It consists in the use of *mixtures* of sulfonamides instead of single compounds. The idea emerged from the observation that a saturated aqueous or urinary solution of a sulfonamide could still be fully saturated with a second and third sulfonamide of different molecular structure, each compound behaving as though it were present alone and exerting no influence on the solubilities of the others. Consequently, in solutions containing several sulfonamides, the maximum obtainable concentration appeared expressed by the sum of the solubilities of all the drugs present. This finding applied to the free compounds, as well as to their acetylated homologues.

It was reasoned on the basis of this observation that the danger of intrarenal formation of sulfonamide crystals could be considerably reduced by employing combinations of *partial* dosages of 2 or more therapeutically equivalent sulfonamides instead of single compounds. The validity of this contention was demonstrated in experimental and clinical studies with a mixture of sulfathiazole and sulfadiazine.[2,3]

The present paper deals with the toxicity of a sulfadiazine-sulfamerazine and a sulfathiazole-sulfadiazine-sulfamerazine combination. It contains also some additional data on the sulfathiazole-sulfadiazine mixture, in particular a comparative quantitative estimation of intrarenal sulfonamide deposits from sulfathiazole, sulfadiazine, and their combination.

Materials and Methods. Four hundred albino rats from our own colony, 8-12 weeks old and weighing between 160-210 g were employed in all experiments with the exception of the study on chronic toxicity in which weanling rats were used. The animals were kept on a standard diet (Rockland Farms Rat Diet) and had free access to water.

For determination of the acute toxicity, observation was continued for 5 days following a single intraperitoneal injection of the sulfonamides because of the well known de-

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* This investigation has beeen aided by grants from the Josiah Macy, Jr., Foundation and the Schering Corporation, Bloomfield, N.J.

[1] Lehr, D., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 11.

[2] Lehr, D., *J. Urol.*, 1946, **55**, 548.

[3] Lehr, D., Slobody, L. B., and Greenberg, W. B., *J. Pediat.*, 1946, **29**, 275.

TABLE I.
Comparative Acute Toxicity in Male Albino Rats of Sodium Salts of Sulfadiazine (NaSD), Sulfathiazole (NaST), Sulfamerazine (NaSMD), and Mixtures of 2 and 3 of These Compounds.

Drug, g/kg body wt					
Single intraperitoneal inj. of	Sulfonamide total amt	No. of animals	No. of dead	% dead	Died within days
NaSD, 1.5	1.5	90	76	85	2-4
NaST, 1.1	1.1	25	16	65	1-4
NaSMD, 1.5	1.5	29	15	52	1-3
NaSD, 0.75 NaST, 0.55	1.3	60	7	12	2-4
NaSD, 0.75 NaSMD, 0.75	1.5	30	11	37	1-2
NaSD, 0.54 NaST, 0.42 NaSMD, 0.54	1.5	30	1	3	1-2

layed death from sulfadiazine, and to some extent also from sulfamerazine intoxication. As a rule 5% aqueous solutions of the sodium salts of sulfathiazole, sulfadiazine, and sulfamerazine were employed. Mixtures of two drugs were prepared conveniently by mixing equal parts of the 5% solutions so that the final concentration was 2.5% each of sulfadiazine and sulfathiazole, or sulfadiazine and sulfamerazine. If unequal amounts of 2 or 3 drugs were used, as in the acute toxicity study with the sulfathiazole-sulfadiazine mixture, the initial concentrations of the individual drugs were so adjusted, that mixing of equal fluid volumes resulted in the desired final concentrations of the 2 or 3 components. Thus identical amounts of fluid were injected in all groups. In each test the toxicity of combined sulfonamides was evaluated *simultaneously* with the toxicity of equal or comparable dosages of the separate components of the mixture.

Ten rats represented the minimum experimental unit. Subgroups of 5 animals each were placed into separate metabolism cages. The sulfonamide concentration in the blood was determined from the tail vein of each animal, at least at one occasion, in order to eliminate rats injected inadvertently into the gut instead of intraperitoneally. In some experiments the sulfonamide level in blood and urine was followed for 48 hours.

In other studies groups of animals were killed at predetermined intervals of a 48-hour period. The blood was used for estimation of the sulfonamide and nonprotein nitrogen level. The entire urinary tract was carefully inspected for the presence of crystalline deposits. The kidneys were weighed and cross-sections examined under the lens for concrements in the pelvis and for intratubular drug precipitation. The sulfonamide concentration was then determined in the kidneys according to a procedure previously outlined in detail.[4]

The subacute toxicity was studied by repeated intraperitoneal injections of sublethal dosages of the sulfonamides and their mixtures. The level of nonprotein nitrogen and sulfonamide in the blood was determined repeatedly and the total daily drug excretion in the urine was followed throughout the entire experimental period.

The chronic toxicity was determined by incorporating the sulfonamides in various concentrations and combinations into the powdered form of the standard diet. The experiments were conducted with weanling rats weighing 60-80 g and planned for a period of 6 weeks. The food consumption was checked daily. The body weight of each animal was recorded twice weekly. Blood concentration and total urinary elimination of sulfonamide were determined at the same time intervals. The nonprotein nitrogen levels in the blood were estimated upon termination

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[4] Lehr, D., and Antopol, W., *Urol. and Cutan. Rev.*, 1941, **45**, 545.

TABLE II.

Findings in Blood and Kidneys 24 Hours After a Single Intraperitoneal Injection of Sulfonamides in Albino Rats.

		Blood			Kidney (moist tissue)				
						Total sulfa			
Group No.	Drug, g/kg	N.P.N., mg %	Total sulfa, mg %	% acetylation	Wt (both kidneys)	mg %	mg	% acetylation	Appearance of renal cross-section
1	NaSD, 1.5	197	140	19	2287	454	10.3	23	Considerable amt of drug ppt. in entire medulla. Marked edema.
2	NaST, 1.1	154	62	27	2137	160	3.4	40	Same in the papilla and pelvis. Marked edema.
3	NaSD, 0.75 NaST, 0.55	104	30	7	1763	34	0.6	6	No ppt. visible in 2 animals. Trace of ppt. in 1 animal. Slight edema.
4	NaSD, 0.75	143	51	22	2259	89	2.0	38	Ppt. obstructs the papilla. Marked edema.
5	NaST, 0.55	53	1	23	1436	0	0	0	No ppt. visible. No edema.
6	Controls	48			1472				

All figures represent the mean of the values from 3 female animals.

of each experiment and correlated with the pathologic-anatomical findings in the kidneys.

In all experiments, the surviving rats were killed by exsanguination in ether narcosis. Postmortem examinations were performed on these animals and as far as feasible also on rats succumbing during an experiment. Special attention was given to changes in the urinary tract. The most important organs of representative animals were fixed in formaldehyde for histological study.

Determination of free and conjugated sulfonamide was carried out according to the method of Bratton and Marshall[5] using a Klett Summerson photo colorimeter. All figures of drug concentration were expressed in terms of the free sulfonamides and not of their sodium salts. Obviously no differentiation could be made between sulfathiazole, sulfadiazine and sulfamerazine in determinations of mixtures in body fluids. However, these compounds have very similar molecular weights and, therefore, give almost identical colorimetric readings.

Results. 1. *Acute Toxicity.* A condensation of the most important results from the acute toxicity studies is presented in Tables I and II and in Fig. 1.

Sulfadiazine-Sulfathiazole Mixture. It is apparent from Table I that the extensive increase in the number of animals in the sulfathiazole-sulfadiazine experiment did not result in any significant change in the mortality figures as compared to those originally reported.[1]

It was inferred from these results that the danger of renal blockage can be significantly reduced by the joint administration of sulfadiazine and sulfathiazole in *partial* dosages. Added proof for the validity of this viewpoint was derived from a series of experiments conducted to determine the accurate amounts of sulfonamide present in the kidneys 4 hr., 8 hr., 24 hr., and 48 hr. after a single intraperitoneal injection of the sulfadiazine-sulfathiazole mixture as compared to either compound when injected separately.

It was found that intrarenal precipitation

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[5] Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.

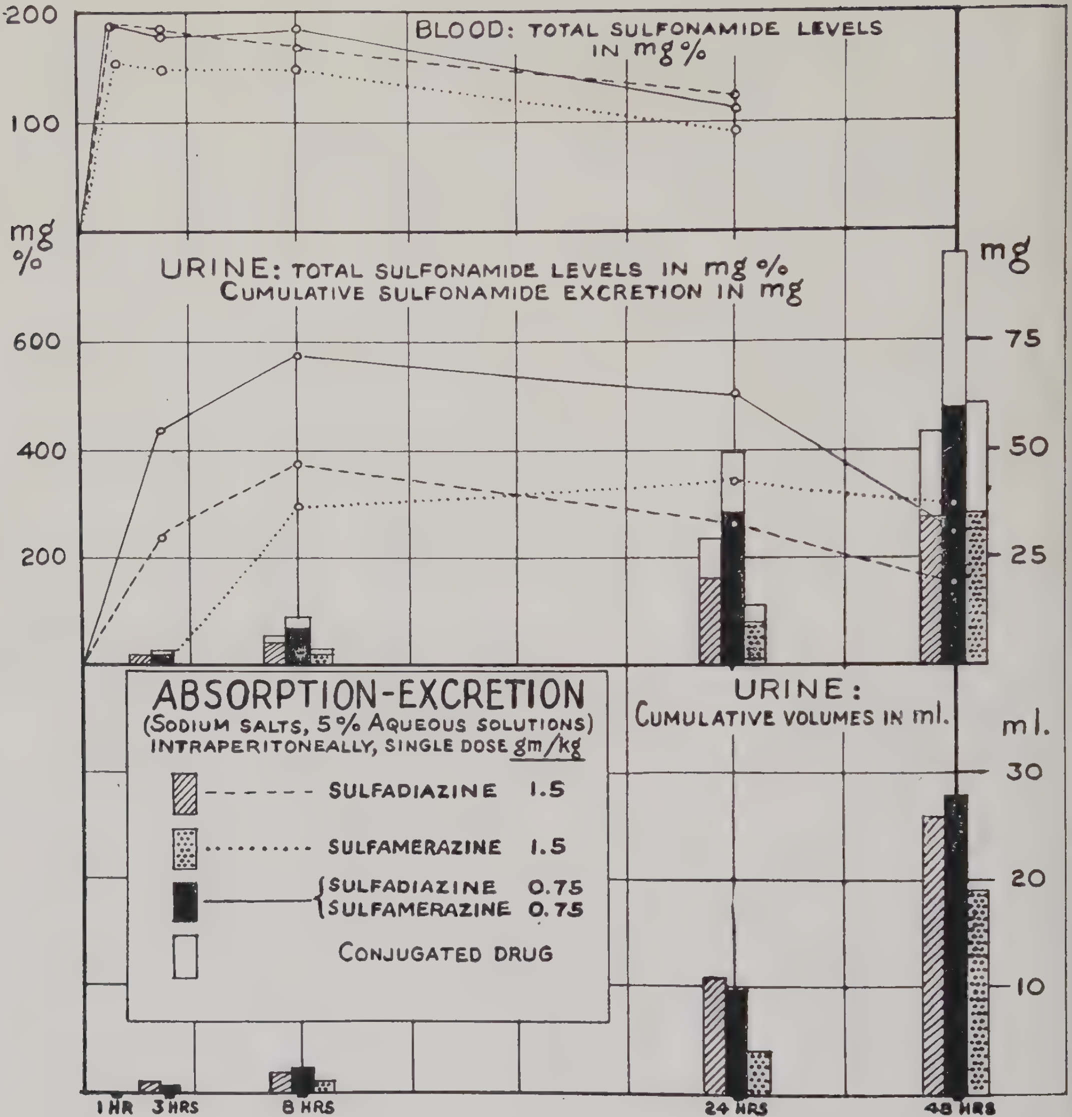

Fig. 1.

Graphic representation of the comparative absorption and excretion of sulfadiazine, sulfathiazole and a mixture of these two compounds in albino rats.

of sulfonamide crystals occurred initially in all groups, even in those receiving only *half* the dosages of either sulfathiazole or sulfadiazine alone. But there were highly significant differences in the quantity and duration of intratubular crystalline deposition.

Table II summarizes one representative experiment. The table demonstrates that after 24 hours the rats injected with the sulfadiazine-sulfathiazole mixture (Group 3) were practically free of renal drug deposits. Actually the kidneys of these animals contained less than one-tenth the amount of sulfonamide expected on the basis of the values from the full dosages of the separate compounds (italic figures), and even this small amount is fully accounted for by the general tissue concentration, as apparent from the sulfonamide blood level.

It was interesting to note that half the dose of sulfadiazine resulted in more precipitation when administered separately than

TABLE III.
Urinary Elimination of Single and Mixed Sulfonamides After One Intraperitoneal Injection in Albino Rats.
(Each value represents the mean from at least 10 animals.)

Drug	Dose, g/kg		% of administered dose excreted with urine in:		
	Partial	Total	8 hr	24 hr	48 hr
NaSD		1.5	1.9	9.6	16.0
NaSMD		1.5	1.2	5.4	24.0
NaSD	.75	1.5	4.7	21.0	40.0
NaSMD	.75				
NaSD	.54	1.5	9.0	39.0	50.0
NaSMD	.54				
NaST	.42				

TABLE IV.
Comparative Subacute Toxicity in Male Albino Rats of Sodium Salts of Sulfadiazine (NaSD), Sulfathiazole (NaST), Sulfamerazine (NaSMD), and Mixtures of 2 and 3 of These Compounds. (The mortality figures are shown as they appeared when 100% mortality was reached in the sulfathiazole group, that is, after 4 injections.)

Drug, g/kg body wt					
Daily intraperitoneal inj. of		Sulfonamide total amt. inj.	No. of animals	% death	Died within days
NaSD,	.9	3.6	15	87	2-5
NaST,	.9	3.6	15	100	1-4
NaSMD,	.9	3.6	20	55	3-5
NaSD,	.45	3.6	10	30	3-4
NaST,	.45				
NaSD,	.45	3.6	10	0	
NaSMD,	.45				
NaSD,	.3	3.6	10	0	
NaST,	.3				
NaSMD,	.3				

when given in combination with sulfathiazole. This paradoxical phenomenon can be explained by the greater diuresis in the presence of higher sulfonamide concentrations in the tubular urine. It is obvious that sulfonamide mixtures retain the same osmotic value as equal concentrations of single compounds. Thus animals receiving the partial dose of only *one* component of the mixture are exposed to no less danger of oversaturation in the tubular urine, while at the same time they are deprived of the greater diuretic effect of higher sulfonamide concentrations as present in the urine of mixture animals.

Mixtures of Sulfadiazine-Sulfamerazine and Sulfadiazine-Sulfathiazole-Sulfamerazine. The low toxicity of these 2 combinations is illustrated in Table I. It can be seen that a mixture of 3 sulfonamides is significantly less toxic than a mixture of 2 compounds.

The absorption and excretion of sulfonamide combinations is exemplified in Fig. 1. It is evident that a mixture of half dosages of sulfadiazine and sulfamerazine is more completely absorbed and excreted than either compound administered separately in the same total dosage. Similar observations were made with the mixture of sulfadiazine-sulfathiazole and the combination of all 3 sulfonamides. The latter gave the most complete urinary recovery figures.

In this connection, it should be kept in mind that excessive dosages of sulfonamides, which result in renal obstruction, are neces-

TABLE V. Body Weight, Nonprotein Nitrogen and Sulfonamide Levels in Blood, and Pathologic-Anatomical Findings in Kidneys of Albino Rats Surviving After 6 Weeks on a Diet Containing Either Sulfadiazine, Sulfamerazine, or a Mixture of the 2 Drugs.

% drug in the diet	No.	Sex	Body wt	Blood N.P.N. mg %	Blood Sulfonamide, mg %	Findings in the kidneys	Remarks
3% SD	1	F	88	103	33	Parenchymatous damage in all survivors with calcifying nephrosis in 3 instances	1 died within 5 wk
	2	F	76	101	24		
	3	F	93	145	37		
	4	F	76	121	37		
	5	F	80	126	31		
	6	M	115				
	7	M	110	149	34		
	8	M	108	93	30		
	9	M	113	107	27		
		Avg	95	118	31		
3% SMD	2	F	63	89	56	Many concrements throughout entire urinary tract in one survivor; gravel in the pelves and intrarenal precipitate in the other four	4 died within 4 wk
	5	F	76	103	57		
	8	M	84	110	53		
	9	M	60	84	69		
	10	M	80	64	54		
		Avg	73	90	58		
1½% SD 1½% SMD	1	F	78	70	64	Traces of gravel in 4 of 9 survivors; otherwise normal kidneys	1 died within 2 wk
	2	F	75	45	55		
	3	F	77	84	45		
	4	F	68	67	64		
	5	F	78	45	49		
	7	M	84	75	62		
	8	M	74	73	63		
	9	M	80	72	63		
	10	M	88	61	64		
		Avg	78	66	59		
Controls	1	F	148	53			
	2	F	144	47			
	3	F	166	44			
	4	F	150	46			
	5	F	160	34			
	6	M	248	45			
	7	M	202	58			
	8	M	192	54			
	9	M	200	46			
	10	M	215	50			
		Avg	183	48			

sarily much less completely eliminated with the urine than small amounts. Hence high recovery figures in the urine, as observed with sulfonamide combinations, can mean only diminished interference with renal elimination. In line with this interpretation, Table III indicates that the percentage of urinary recovery is directly proportional to the number of sulfonamides in the combination, since any addition to this number results necessarily in smaller partial dosages of each component.

2. *Subacute Toxicity.* The results of the studies in male albino rats are summarized in Table IV. All 3 sulfonamide combinations are again conspicuous because of their low toxicity.

A comparison with Table I reveals that the results of the subacute experiments were in good agreement with those of the acute study. This applied also to levels of nonprotein nitrogen and sulfonamide in the blood as well as to urinary drug excretion and autopsy findings. Identical acute and subacute toxicity studies with *female* rats were confirmatory, although female animals proved in general distinctly more sensitive to the sulfonamides.

3. *Chronic Toxicity.* Studies with a sulfathiazole-sulfadiazine food mixture had to be abandoned because weanling rats submitted to almost complete voluntary starvation at sulfathiazole and at sulfathiazole-sulfadiazine concentrations of 5% and even 3% in the diet.

Sulfamerazine, although still less well tolerated than sulfadiazine, permitted an intake at the 3% level in the food sufficient both for the survival of the rats and for the creation of serious renal lesions. It should be stressed that at the same concentration the sulfadiazine food was consumed in larger amounts; accordingly, the voluntary intake of the sulfadiazine-sulfamerazine combination was in between the one of either compound given separately. The end result of a 6-week feeding study is shown in Table V.

Measured in terms of survival and inhibition of growth, sulfamerazine would appear to be more toxic than sulfadiazine and the combination. In comparing sulfadiazine and the sulfadiazine-sulfamerazine mixture one might be misled by the greater retardation of growth from the latter into considering the mixture more toxic than sulfadiazine. However, if one compares the nonprotein nitrogen and sulfonamide levels in the blood as well as the findings in the kidneys, it becomes immediately apparent that sulfadiazine like sulfamerazine is by far more toxic than the combination of the 2 drugs.

The less significant growth inhibition in the sulfadiazine group was obviously due to the higher food consumption. Despite the simultaneously greater drug intake, the blood levels from sulfadiazine were throughout the experiment lower than from sulfamerazine and the combination. Since there was little difference in the total urinary excretion of sulfadiazine and sulfamerazine, it is obvious that sulfadiazine was slower and less completely absorbed from the gastro-intestinal tract and faster excreted by the kidneys than sulfamerazine. Renal damage was very marked in the sulfadiazine as well as in the sulfamerazine group, whereas it was insignificant in the animals fed the combination of the 2 drugs.

The fact that sulfamerazine may produce more concrement formation than a higher dose of sulfadiazine should serve as a warning that renal complications may occur from sulfamerazine at least as readily as from sulfadiazine. Although this finding is in contrast to previous experimental observations,[6,7] it is strongly supported by growing clinical experience.[8,9]

Discussion. The present study extended the experimental evidence for the strikingly low toxicity of sulfonamide combinations from the sulfathiazole-sulfadiazine mixture

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[6] Welch, A. D., Mattis, P. A., Latven, A. R., Benson, W. M., and Shields, E. H., *J. Pharm. and Exp. Therap.*, 1943, **77**, 357.

[7] Schmidt, L. H., Hughes, H. B., and Badger, E. A., *J. Pharm. and Exp. Therap.*, 1944, **81**, 17.

[8] Flippin, H. F., and Reinhold, J. G., *Ann. Int. Med.*, 1946, **25**, 433.

[9] Hageman, P. O., Harford, C. G., Sobin, S. S., and Ahrens, R. E., *J. A. M. A.*, 1943, **123**, 325.

to include combinations of sulfadiazine-sulfamerazine, and sulfathiazole-sulfadiazine-sulfamerazine. Confirmatory experimental results were reported recently by other investigators.[10,11] In this connection it is of importance to stress that the toxicity of sparingly soluble sulfonamides is dependent upon 2 factors. The one, representing the true tissue toxicity, consists in the direct chemical action of the sulfonamides on the living cell; the other, as a function of solubility and renal clearance of these compounds, lies in the hazard of mechanical blockage in the urinary tract.

Lately the factors influencing the prevention and control of renal blockage from sulfonamides were summarized in a lucid review by Scudi.[12] The significance of renal obstruction in the over-all toxicity of heterocyclic derivatives of sulfanilamide was demonstrated in the animal experiment.[4,13] It was also shown that measures which diminish the intrarenal formation of sulfonamide crystals[14] or remove deposits from the tubules[15] will result in a most significant drop in the mortality. The low toxicity of sulfonamide combinations can, therefore, be interpreted in the light of experimental proof for a strongly diminished tendency to intrarenal precipitation, as due mainly to a decrease in the renal factor of toxicity. This viewpoint is strengthened by the lack of evidence for any other mechanism of detoxification, since one would not expect pronounced changes in the true cellular toxicity of closely related compounds if they retain their full antibacterial activity in combinations.

In line with this interpretation was the further significant lowering of the toxicity for a mixture of *3* sulfonamides. It was obviously due to the fact that the triple mixture contained necessarily *smaller* partial dosages of the individual compounds, resulting in a further diminution of the possibility of oversaturation and hence precipitation in the tubular urine.

Since at the bedside crystalluria was strongly decreased, even with mixtures of *2* sulfonamides and in the absence of alkalization,[2,3,8] it seems justified to assume that one might safely dispense with the burden and disadvantages[8,16] of adjuvant alkali therapy when employing combinations of *3* sulfonamides.

The unexpectedly high blood and urine levels obtained with mixtures, as well as the significantly greater recovery from the urine as compared to single sulfonamides, can be explained by a more complete absorption of mixtures and the greater ease of their renal elimination. These findings were confirmed in extensive absorption and excretion studies with sublethal dosages of sulfonamide combinations administered by the intraperitoneal and oral route. They proved that for any given sulfonamide the completeness of absorption and urinary elimination was, within limits, inversely proportional to the size of the dose used. This behavior remained unchanged for each drug if several different sulfonamides were administered simultaneously. In other words, *in mixtures of sulfonamides the body handles each compound as if it were present alone and in the amount contained in the combination.*

With regard to *in vitro* antibacterial activity, it was reported previously[1] that the sulfathiazole-sulfadiazine combination showed essentially an additive effect and that in some instances the combination was even more effective than either compound alone in equal concentration. Continuation of these experiments rendered identical results for a mixture of sulfadiazine and sulfamerazine, and a combination of all 3 compounds. However, it should be remembered that these observations were made in the test tube with equal sulfonamide concentrations, whereas

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[10] Whitehead, R., Sect. Exp. Med. and Therap., A.M.A. Meeting, San Francisco, July 3, 1946.

[11] Frisk, A. R., Hagerman, G., Helander, S., and Sjogren, B., *Nordisk Med.*, 1946, **29**, 639.

[12] Scudi, J. V., *Am. J. M. Sc.*, 1946, **211**, 615.

[13] Lehr, D., Antopol, W., and Churg, J., *Science*, 1940, **92**, 434.

[14] Lehr, D., *Bull. New York Acad. Med.*, 1944, **20**, 424.

[15] Lehr, D., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 82.

[16] Beyer, K. H., Peters, L., Patch, E. A., and Russo, H. F., *J. Pharmacol.*, 1944, **82**, 239.

the administration of equal dosages of the 3 sulfonamides and their combinations would result in greatly different drug concentrations in the living body. In accordance with the results of absorption-excretion studies, as exemplified in Fig. 1, sulfonamide mixtures give high and well sustained blood concentrations and should, therefore, prove at least of the same if not of higher antibacterial value when compared with the same dosage of any one of their individual components *in vivo*. Although this viewpoint is well supported by clinical experience,[2,3] it remains to be accurately investigated in therapeutic studies with experimental infections of laboratory animals.

Summary. 1. In continuation of experimental and clinical studies with mixtures of sulfonamides, the toxicity as well as the absorption and excretion of the combinations sulfadiazine-sulfathiazole, sulfadiazine-sulfamerazine, and sulfadiazine-sulfathiazole-sulfamerazine were investigated in albino rats.

2. These combinations of partial dosages proved significantly less toxic than any one of their separate constituents in equal or comparable total dosage. The mixture of 3 sulfonamides was less toxic than either combination of 2 drugs.

3. The low toxicity of sulfonamide mixtures was shown to be due to the prevention of renal obstruction, resulting from a pronounced diminution in the intratubular deposition of sulfonamide crystals.

4. Mixtures of sulfonamides were more completely absorbed and excreted than equal amounts of their individual constituents. Blood levels from mixtures were, therefore, distinctly higher than expected on the basis of mathematical computations from the values of single sulfonamides.

5. It was reasoned on the basis of these experimental studies that the use of a mixture containing 3 sulfonamides in human therapy would almost completely eliminate the possibility of concrement formation in the urinary tract at the routine dose level. Hence, it would also obviate the necessity for adjuvant alkali therapy.

The technical assistance of the Misses Helen and Ruth Salzberg and Miss Catherine Russell is gratefully acknowledged.

15807

Surface Striations of *Euglena gracilis* Revealed by Electron Microscopy.

VINCENT GROUPÉ. (Introduced by Geoffrey Rake.)

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In the course of studying a variety of microorganisms with the aid of electron microscopy using the shadow casting technic of Williams and Wyckoff[1] and the replica technic of Hillier and Baker[2] a definite pattern of surface striations on the pellicle of *Euglena gracilis* was clearly revealed. The presence of such surface striations on this species suggests the possibility that many or all of the species of *Euglena* possess these markings in varying degree inasmuch as prominent surface striations have been described for other species of *Euglena (e.g., E. viridis, E. oxyrus,* and *E. spirogyra.)*[3]

Bacteria-free cultures of 4 physiologically different species of *Euglena gracilis,* obtained through the courtesy of Dr. George W. Kidder, were maintained by serial passage on

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[1] Williams, R. C., and Wyckoff, R. W. G., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 265.

[2] Hillier, J., and Baker, R. F., *J. Bact.*, 1946, **52**, 411.

[3] Kudo, R. R., *Handbook of Protozoology,* Charles C. Thomas, Springfield, Ill., 1931, 117-120.

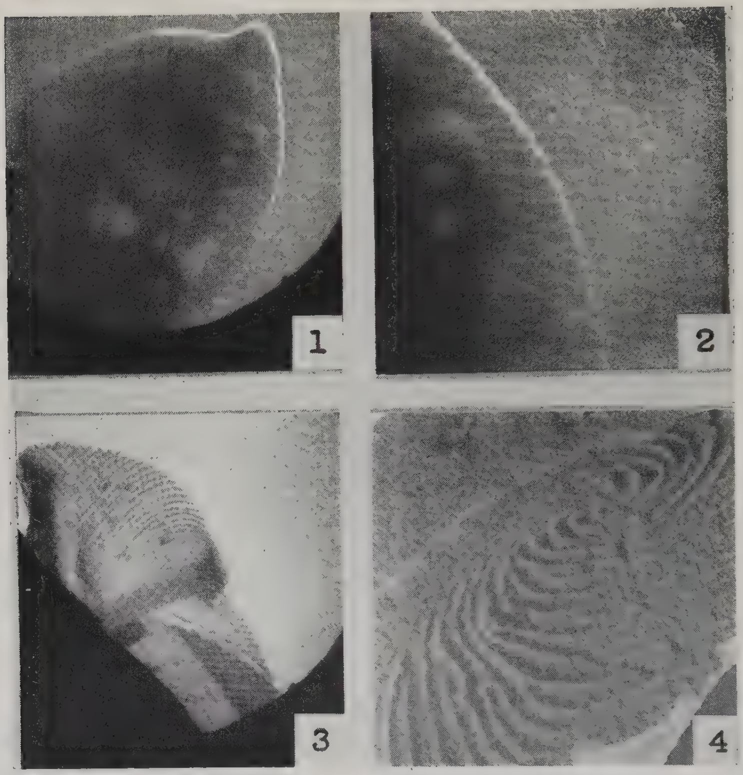

FIG. 1-4.
Electron Micrographs of *Euglena gracilis* Shadowed with Gold.
FIG. 1. Shadowed with 22.0 mg gold at the angle tangent 3/8.5. Magnification 3370 ×.
FIG. 2. Shadowed with 22.0 mg gold at the angle tangent 3/8.5. Magnification 7800 ×.
FIG. 3. Shadowed with 21 mg gold at the angle tangent 3/8.5. Magnification 3370 ×.
FIG. 4. Shadowed with 24 mg gold at the angle tangent 3/9. Magnification 8530 × (collodion replica).

tryptone acetate broth. Organisms for study were obtained from plate cultures of the various strains on tryptone acetate agar and were prepared for examination in the electron microscope using the replica technic of Hillier and Baker.[2] Specimens thus prepared were shadowed with gold according to the technic of Williams and Wyckoff[1] using a shadow casting device designed and built by Dr. H. Sidney Newcomer. An RCA electron microscope (Type EMU) was used throughout these studies.

It will be seen from the pictorial data presented in Fig. 1 and 2 that groove-like striations forming a regular pattern are present on the surface of *Euglena gracilis*. That these striations are located on the pellicle is evident from the micrograph presented in Fig. 3. It will be seen that the cell has ruptured allowing the protoplasm to escape and had left the pellicle virtually intact thus clearly revealing the pattern of striations on the membrane. Furthermore, it would appear that the striations originate from a central point located on the side of the pellicle and spiral outward and around the organism producing a pattern suggestive of a fingerprint. The structure of such a central point is shown in greater detail in the collodion replica of a portion of the surface of an organism presented in Fig. 4. A similar pattern of surface striations was observed on each of the 4 strains of *Euglena gracilis* studied.

Unfortunately, no intact flagella were observed and, although replicas of flagella were occasionally encountered, no evidence of structural differentiation was seen.

Summary. A regular pattern of groove-like striations was observed on the pellicle of *Euglena gracilis.*

15808

In vitro Sensitivity of *Brucella* to Streptomycin: Development of Resistance During Streptomycin Treatment.*

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Like many other species of bacteria brucella are remarkably sensitive *in vitro* to the action of streptomycin.[1-5] Experimental brucella infections in animals[1,3] have also been favorably influenced by the administration of streptomycin. However, the use of streptomycin in human brucella infections has not been attended by consistently favorable results.[4-8] The cause of this has not been elicited and there have been no reports in the literature of the development of resistance during the treatment of human brucella infections with streptomycin. The present report summarizes the results of numerous tests of the *in vitro* sensitivity of the 3 varieties of brucella to streptomycin. Studies are reported concerning a strain of *Br. abortus* which developed marked resistance to the action of streptomycin during the treatment of a patient having subacute bacterial endocarditis due to this organism.

Methods and Materials. The method of testing the *in vitro* sensitivity of 40 strains of brucella was as follows: a loopful of a 24-hour culture grown on a Bacto tryptose phosphate agar† slant was transferred to 10 ml of sterile tryptose phosphate broth so as to give a suspension with a turbidity equal to that of a barium sulfate No. 1 standard. This suspension was then serially diluted to 10^{-3} with tryptose phosphate broth. Pour plate colony counts indicated that this diluted suspension contained 30,000 to 300,000 viable cells per ml. Each of a series of 10 test tubes was inoculated with 4.4 ml of tryptose phosphate broth. To each of 9 tubes was added 0.1 ml of the 10^{-3} dilution of brucella. The 10th tube was used as a sterility control. To all but the sterility control was added 0.5 ml of streptomycin dissolved in sterile physiological saline, the amount of streptomycin added being sufficient to give a final concentration of 0.5 to 10 μg of streptomycin base per ml. The streptomycin solution

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* This study was supported by grants from the American Medical Association and the United States Public Health Service and was carried out in the Laboratories of the Minnesota State Department of Health. The streptomycin was supplied by the Committee on Chemotherapeutic and Other Agents of the National Research Council.

[1] Jones, D., Metzger, H. J., Schatz, A., and Waksman, S. A., *Science*, 1944, **100**, 103.

[2] Waksman, S. A., and Shatz, A., *J. Am. Pharm. A. (Scient. Ed.)*, 1945, **34**, 237.

[3] Live, I., Sperling, F. G., and Stubbs, E. L., *Am. J. M. Sc.*, 1946, **211**, 267.

[4] Herrell, W. E., and Nichols, D. R., *Proc. Staff Meet., Mayo Clin.*, 1945, **20**, 449.

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[6] Nichols, D. R., and Herrell, W. E., *J. A. M. A.*, 1946, **132**, 200.

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[8] Hall, W. H., Braude, A., and Spink, W. W., *Staff Meet. Bull. Hosp. of Univ. of Minn.*, 1946, **18**, 109.

† Difco Laboratories, Detroit.

was preserved in the frozen state in a dry ice freezing cabinet and showed no decline in activity as a result of storage for several weeks. Commercial streptomycin in the form of the sulfate or hydrochloride from several different producers was used. Each lot of streptomycin was checked for potency against a sensitive strain of *Br. abortus*. After the addition of the streptomycin each tube was mixed thoroughly by rotation. The tubes were then incubated at 37°C. Unless otherwise stated the strains of *Br. abortus* were incubated under 10% CO_2. After 24 hours incubation each tube was mixed and one standard loopful was subcultured on a tryptose phosphate agar plate. The plates were incubated at 37°C (*Br. abortus* under 10% CO_2) and examined for colonies of brucella with a hand lens after 96 hours.

The majority of the brucella cultures were tested for streptomycin sensitivity within a few days after they had been isolated from the blood of patients. In some cases the cultures had been suspended in 10% horse serum and stored in the frozen state for several months. After a few transfers on tryptose phosphate agar each culture was tested to ascertain that it was smooth and had the biochemical characteristics of one of the 3 varieties of brucella. We had the opportunity of testing the sensitivity to streptomycin of cultures of *Br. abortus* isolated from the blood of 3 patients before, during and/or after streptomycin therapy.

TABLE I.
Sensitivity of Brucella to Streptomycin *in vitro*.

Variety of brucella, and strain	Max. conc. permitting growth, μg/ml	Min. conc. completely inhibiting growth, μg/ml
Brucella abortus		
Stock	0.5	1
83	1	2
165	—	0.5
94	2	3
98	1	2
104	2	3
110	2	3
132	1	2
372	1	2
419	1	2
424	1	2
439	1	2
449	1	2
467	2	3
483	1	1.5
484	3	4
488	0.5	2
490	2	3
495	1	2
524	0.5	2
552	0.5	1
607	1	2
Grono (Rough)	50	100
Rowe 11-9	0.5	1
Shadick 11-5	1	1.5
Elsted 9-28	0.5	1
Brucella suis		
Stock	1	2
67	0.5	1
156	1	2
263	1	2
303	0.5	1
306	0.5	1
346	0.5	1
456	0.5	1
37	2	3
80	1	2
88	1	2
145	0.5	1
616	0.5	1
Brucella melitensis		
Stock	1	2

Results. In Table I are given the concentrations of streptomycin which completely inhibited the growth of 26 cultures of *Br. abortus*, 13 cultures of *Br. suis*, one stock culture of *Br. melitensis* (of unknown origin) and one stock rough culture of *Br. abortus*. None of the patients had received streptomycin therapy prior to the isolation of the organisms. Inspection of Table I reveals that all the strains of brucella were very sensitive to the action of streptomycin *in vitro* with the exception of the rough stock culture of *Br. abortus*. At first glance strains of *Br. suis* appear to be more sensitive than most strains of *Br. abortus*. The difference lies in the fact that incubation under an atmosphere containing 10% carbon dioxide reduces the sensitivity of brucella to streptomycin. Strains of *Br. abortus* which had been adapted to aerobic growth increased in sensitivity to streptomycin when the test was carried out aerobically. When strains of *Br. suis* were tested for streptomycin sensitivity in an atmosphere containing 10% carbon dioxide streptomycin proved to be less effective in inhibiting their growth. The magnitude of the difference is shown in Table II.

It was also found that the size of the

TABLE II.
Effect of Carbon Dioxide on *in vitro* Sensitivity of Brucella to Streptomycin.

Variety of brucella and strain	Minimum concentration of streptomycin completely inhibiting growth, μg/ml	
	Aerobic conditions	10% CO_2 added
Brucella abortus		
439	1	2
552	0.5	1
Brucella suis		
37	2	3
80	2	3
88	2	3
145	1	3
616	1	3

inoculum affected the concentration of streptomycin necessary to completely inhibit growth *in vitro*. The growth of strain 524 of *Br. abortus* was completely inhibited by 1 μg per ml when an inoculum of 30,000 cells was used but 4 μg per ml was necessary when the inoculum was 30,000,000 cells. The concentration of streptomycin necessary to completely inhibit the growth of brucella is also dependent upon the time factor. One loop subcultures taken at 24-hour intervals revealed that after 48 hours exposure to streptomycin 15 of 20 strains of *Br. abortus* were inhibited by a concentration of streptomycin equal to 50% of that necessary to inhibit their growth after only 24 hours exposure. Subcultures at the end of 3 and 7 days revealed no further apparent increase in sensitivity. Similar observations were made with 3 of 7 strains of *Br. suis* and one strain of *Br. melitensis*. Furthermore there was no evidence that prolonged incubation of brucella in contact with streptomycin promoted the growth of streptomycin-resistant variants.

It is of interest that in 2 patients with chronic brucellosis and bacteremia who failed to benefit from streptomycin therapy there was no evidence that this failure was due to the development of streptomycin-resistant organisms. Nor was there any evidence that the presence of human serum reduced the *in vitro* sensitivity of these strains of *Br. abortus* to streptomycin. The first of these patients (P.L.) received 19 g of streptomycin intramuscularly in 9 days. Strain 424, *Br. abortus*, was isolated from her blood stream before treatment and proved to be sensitive to 2 μg of streptomycin per ml. Strain 524 was isolated from her blood after 8 days of streptomycin therapy and was equally sensitive. The second patient (A.S.) was given 27.6 g of streptomycin intramuscularly in 9 days. Strain 483, *Br. abortus*, was recovered from his blood before streptomycin therapy and was inhibited by 1.5 μg streptomycin per ml. Four months later strain 11-5 was isolated from his blood, and it showed no decrease in streptomycin sensitivity.

However, in a third patient (K.E.) streptomycin resistance did develop during streptomycin therapy. This patient was a young farmer who developed acute brucellosis in 1944. His symptoms remitted with sulfonamide and vaccine therapy. However, his symptoms recurred 2 years later and were accompanied by signs of subacute bacterial endocarditis. *Br. abortus* strain 9-28 was isolated from his blood culture; it proved to be sensitive to 1.0 μg streptomycin per ml. He was then given 118 g of streptomycin intramuscularly over a period of 31 days. Strain 11-6 was isolated from his blood on the 29th day of streptomycin therapy. This strain grew in the presence of 7,500 μg of streptomycin per ml but its growth was inhibited in the presence of 10,000 μg per ml. *Br. abortus* was isolated from his blood 4 and again 5 days later; these cultures were equally resistant to streptomycin *in vitro*. One loop subcultures revealed the presence of a few viable brucella when these resistant cultures were exposed for 24 hours to concentrations as high as 50,000 μg of streptomycin per ml *in vitro*. The patient was given large doses of sulfadiazine orally for 2½ months at the conclusion of his streptomycin therapy and at the present time his infection is apparently arrested.

The streptomycin-resistant strain differed from strain 9-28 in several particulars. It grew much more slowly on tryptose phosphate agar and in tryptose phosphate broth. Its growth was particularly slow in the depth of tryptose phosphate agar pour plates, colonies not being visible until the end of 8 to 11 days of incubation. Two distinct colony forms were noted: (1) A large colony type growing relatively rapidly, producing colonies 2 to 3 mm in diameter in 48 hours. These colonies had a smooth surface and were very sensitive to the bactericidal action of human serum but grew in as much as 50,000 μg of streptomycin per ml. Gram stains revealed typical coccobacilli of uniform size and eosinophilic staining properties. They did not ferment the usual sugars and were agglutinated to a high titer by antibrucella rabbit serum. This colony type formed only a small percentage of the total bacterial population. (2) A small colony form was much more numerous. Minute colonies 0.1 mm in diameter appeared in large numbers 4 to 5 days after the resistant strain was streaked on the surface of a tryptose phosphate agar slant. They died quickly unless the inoculum was heavy. They were relatively resistant to the bactericidal action of human serum but would not grow *in vitro* in the presence of more than 7,500 μg of streptomycin per ml. Gram stains revealed a mixture of bizarre, tiny, amorphous, coccoid organisms staining only faintly red and large, dark red, coccoid forms. The small colony variant also had all the serological and biochemical characteristics of *Br. abortus*. Both colony types were smooth and grew only in the presence of added carbon dioxide.

The resistance of strain 11-6 to streptomycin has not changed over a period of 3 months; during this period it has been transferred frequently on tryptose phosphate agar slants. This strain was streaked across the surface of a tryptose phosphate agar plate containing a heavy inoculum of sensitive *Br. abortus*, strain 524 and 3 μg streptomycin per ml. No satellite colonies of strain 524 appeared adjacent to the growth of the streptomycin-resistant strain. It was concluded that the resistant strain did not produce an extracellular, soluble, diffusible streptomycin inhibitor. The apparent resistance of this strain *in vitro* decreased as the length of exposure to streptomycin was increased. Thus subcultures at the end of 7 days incubation revealed that the large colony form was inhibited by 2,500 μg per ml but the small colony variant remained resistant to 7,500 μg per ml. It is of interest that the *in vitro* growth of the resistant strain was stimulated by the addition of sublethal concentrations of commercial streptomycin. The stimulation of growth was particularly noticeable with concentrations of 50 to 1,000 μg of streptomycin per ml. This phenomenon was observed in tryptose phosphate broth, meat infusion broth, tryptose phosphate agar and human serum. The growth of the small colony form appeared to be stimulated more than that of the large colony form. No conclusive evidence was obtained indicating that any similar phenomenon occurs when sensitive strains of *Br. abortus* were exposed to sublethal concentrations of streptomycin.

Discussion. With the exception of one rough strain, all the brucella cultures tested proved to be very sensitive *in vitro* to streptomycin. Brucella appear to develop streptomycin resistance *in vivo* only after prolonged streptomycin treatment. The situation is analogous to that which obtains with *M. tuberculosis* and is presumably due to the slow rate of growth of brucella. It does not appear that the development of resistance to streptomycin by brucella can be the explanation for the frequent therapeutic failures which have been observed after relatively short periods of streptomycin therapy in human brucellosis. While better therapeutic results might follow more extended periods of streptomycin therapy, the possibility of the ensuing development of streptomycin-resistant strains of brucella must also be considered. It is reassuring however that one such streptomycin-resistant strain of *Br. abortus* was less refractory to the bactericidal action of human blood than its sensitive progenitor and remained sensitive to sulfadiazine.

Summary. 1. A method for the *in vitro* testing of sensitivity of brucella to streptomycin is described. 2. The streptomycin sensitivity of 40 cultures of brucella isolated from humans is given. 3. Studies are reported concerning a strain of *Br. abortus* which developed marked resistance to streptomycin during the treatment of a patient having subacute bacterial endocarditis.

15809

Serological Groups and Types of Hemolytic Streptococci Isolated in Puerto Rico.*

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One hundred and sixteen pairs of tonsils were studied bacteriologically. The purulent exudate from the crypts and the inside of each pair of tonsils were cultured separately utilizing methods previously described[1] and the hemolytic streptococci obtained from these tonsils were studied serologically. The distribution of these cultures among Lancefield's groups is given in Table I. The results of a similar study of hemolytic streptococci from normal throats,[2] carried out in 1943-1944, is also included in Table I for purposes of comparison.

The tonsils yielded 62.8% cultures of β-hemolytic streptococci as compared with 17.4% positives among the normal throats. This difference was due mainly to a much higher percentage of Group A organisms obtained from tonsils (30.2%) than from normal throats (1.2%). The proportion of tonsil strains belonging to groups other than A was only twice as large (32.6%) as that obtaining among the throat cultures (16%).

Group B streptococci were obtained in 6% of the tonsils examined. No Group B strains were isolated from the normal throats, but there exists the possibility of these being overlooked if present in sparse numbers.

The distribution of the different Lancefield's groups among the aerobic tonsillar flora is given in some detail in Table II.

When the distribution of strains of different Lancefield's groups cultured from tonsils was compared with the distribution of strains previously obtained from normal throats,[2] it was observed that Group A organisms predominated abundantly in 21 (60%) of the tonsils from which Group A strains were cultured. Contrasting markedly with this, no Group A strain were found in large number among the throats from which A organisms were cultured.

Group C organisms were abundant in 8 (57.1%) of tonsils from which C strains were obtained and in 26 (36.1%) of the normal throats from which C streptococci were isolated.

Group G organisms were obtained in large numbers in 4 (28.4%) and in 5 (16%) respectively of the tonsils and normal throats from which G organisms were cultured.

These figures may suggest that hemolytic streptococci of Groups G and C are of doubtful significance in the production of tonsillitis, however, streptococci belonging to these serological groups are occasionally obtained in abundant numbers and in pure or practically pure culture from diseased tonsils in the absence of any other common pathogen.

No conclusions can be drawn concerning Group B and F due to the small number of strains. It is of interest, however, to observe that these streptococci can be occasionally obtained in pure culture from the pus in the crypts and the inside of tonsils.

Our results agree closely with those reported by Keogh *et al.*[3] in a similar study carried out in Melbourne, Australia.

The serological typing of 95 strains of Group A streptococci obtained from excised

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* This work has been possible through a grant of the insular Department of Agriculture.

[1] Pomales-Lebrón, A., *P. R. J. Pub. Health and Trop. Med.*, 1929, **2**, 196.

[2] Pomales-Lebrón, A., Damin, G. J., Pons, C., and Morales-Otero, P., *P. R. J. Pub. Health and Trop. Med.*, 1946, 287.

[3] Keogh, E. V., MacDonald, I., Battle, J., Simmons, R. R., and Williams, S., *British Med. J.*, 1939, **2**, 1036.

[4] Swift, H. F., Wilson, A. T., and Lancefield, R. C., *J. Exp. Med.*, 1943, **78**, 127.

TABLE I.
Hemolytic Streptococci from 116 Pairs of Excised Tonsils and from 705 Normal Throats.

No. and %	Groups A	B	C	G	F	Total positive	Negative
			Tonsils.				
No. positive	35	7	14	14	3	73	43
% ”	30.2	6	12	12	2.6	62.8	(37.2%)
			Normal Throats.				
No. ”	9	0	72	42	?	123	582
% ”	1.2	0	10.2	6.0	?	17.4	(82.6%)

TABLE II.
Distribtion of Streptococci of Groups A, B, C, G, and F (Lancefield) Among the Aerobic Flora of Excised Tonsils.

Distribution	Cases in which this distribution occurred No.	%
Group A		
Abundantly predominating or in pure culture	21	60.0
Numerous colonies, abundant growth *Staph. aureus*	3	8.6
Few colonies, *Staph. aureus* few colonies	2	5.7
Few colonies; abundant growth *Staph. aureus*	3	8.6
Few colonies; abundant growth of alpha strep.	3	8.6
Moderate growth; moderate growth of alpha strep.	2	5.7
Numerous; few colonies group G	1	2.8
Group B		
Abundant in pure culture	2	28.6
Predominating abundantly in crypts, few colonies of group B inside	1	14.3
Few colonies, abundant growth of *Staph. aureus*	2	28.6
Numerous colonies, few colonies of *Staph. aureus*	1	14.3
Moderate growth, sparse growth of *Staph. aureus*	1	14.3
Group C		
Abundantly predominating or in pure culture	8	57.1
Abundantly predominating in crypts, few group B colonies inside	1	7.1
Few colonies, *Staph. aureus* abundantly predominating	3	21.4
Few colonies and Gram negative cocci predominating	2	14.2
Group G		
Abundantly predominating or in pure culture	4	28.4
Moderate growth, *Staph. aureus* predominating	5	35.7
Predominating, few colonies of *Staph. aureus*	2	14.2
Moderate growth with moderate growth of *Staph. aureus*	2	14.2
Few colonies, abundant growth of group A	1	7.1
Group F		
Abundantly predominating	1	33
Numerous colonies mixed with abundant growth of *Strep. viridans*	1	33
Moderate growth and Gram negative cocci in sparse numbers	1	33

* These figures refer to the percentage distribution of strains of the different groups among the tonsils from which organisms of that particular group were isolated.

tonsils and other sources was attempted utilizing the method described by Swift *et al.*[4] The results are given in Table III.

Representatives of the following types were found among the strains tested: 44, 41, 1, 14, 19, 33, 4, 36 and 39. Approximately one-half of the cultures were negative with all the serums tested.[†] About the same proportion of untypable strains was isolated from the different sources. Types 44 and 41 were the ones more commonly encountered. The number of strains studied being too small no

TABLE III.
Typing of Hemolytic Streptococci from Different Sources.

Source	No. of strains tested	No. and %	Types 44	1	4	41	19	39	33	14	36	Cross reacted*	Negative with serums tested
Excised tonsils	33	No.	8	2	2	2	1	0	0	0	0	3	15
		%	24.3	6.1	6.1	6.1	3.0	0	0	0	0	9.1	45.5
Pathological throats, abscesses, ear and skin infections	22	No.	1	0	0	3	1	1	1	0	0	4	11
		%	4.5	0	0	13.7	4.5	4.5	4.5	0	0	18.2	50
From lesion or affected limb. Recurrent lymphangitis	9	No.	1	0	0	2	0	0	1	0	0	0	5
		%	11.1	0	0	22.2	0	0	11.1	0	0	0	55.5
Normal throats	31	No.	2	3	0	1	3	0	1	1	1	1	18
		%	6.4	9.7	0	3.2	9.7	0	3.2	3.2	3.2	3.2	58.1
Totals	95	No.	12	5	2	8	5	1	3	1	1	8	49
		%	12.6	5.3	2.1	8.4	5.3	1	3.2	1	1	8.4	51.6

* One strain gave a slight precipitate with types 3, 5, 22, 29, 31. Negative with the others. One culture gave a precipitate with No. 28 and No. 44 type serums. The other 6 cross reacting strains gave very slight precipitates with the majority of the type serums.

conclusions can be drawn concerning the distribution of the different types among the various sources.

It must be noted that approximately one-half our strains were untypable. Swift *et al.*[4] state: "negative reactions may also be due to loss of type-specific M substance after isolation of the strain. In some strains and types the M substance remains constant, but in others it may disappear rapidly during subculture on artificial media." The extracts of the strains from excised tonsils were made from stock cultures kept on blood agar slants for many months. The fact that approximately the same proportion of strains from different sources were untypable suggests that this failure was not mainly due to degradation *in vitro* of the organisms with loss of the M substance. There exists the possibility that these strains may be capable of losing their M substance *in vivo*, or may belong to types other than those for which they were tested.

Summary. A bacteriological study of 116 pairs of excised tonsils showed that 62.8% harbored β-hemolytic streptococci. In contrast 705 cultures of normal throats similarly studied showed an incidence of 17.4% of this organism. This difference is due mainly to a higher percentage of Group A streptococci (30.2%) found in excised tonsils as compared to 1.2% in normal throats.

The proportion of streptococci cultured from excised tonsils belonging to groups other than A was twice as large (32.6%) as that obtained from normal throat cultures (16%). In typing 95 strains of hemolytic streptococci by the precipitin method described by Swift *et al.*[4] representatives of types 44, 41, 1, 14, 19, 4, 36 and 39 were found. Approximately half the strains were untypable with the serums tested. Types 44 and 41 were most commonly found.

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† We are greatly indebted to Dr. R. C. Lancefield for supplying type serums and homologous extracts for this study. Extracts from each of our strains were tested with the following type serums, 1, 2, 3, 4, 5, 6, 8, 9, 11, 12, 13, 14, 15, 16, 17, 18, 19, 22, 23, 24, 26, 28, 29, 30, 31, 32, 33, 36, 37, 38, 39, 41, 43, 44, and 46.

15810

Biological Properties and Mouse Virulence of *Streptococcus agalactiae* and Lancefield's Group "B" Streptococci from Human Sources.*

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During a bacteriological study of cow mastitis[1] a large number of strains of *Streptococcus agalactiae* (Lancefield's group B) were isolated. In the course of an investigation of the bacteriology of excised tonsils Lancefield's group B streptococci were isolated from 6% of the tonsils examined.[2] We have occasionally isolated group B streptococci from other pathological conditions. Other workers have isolated group B streptococci from various pathologic processes in humans. The bovine udder has been suspected by some as the ultimate source of group B streptococci from human sources. Careful studies have been made by others[3,4] in an attempt to prove or disprove this point. Further investigation is necessary to find more evidence that might throw light on this problem.

Fifty strains of *Streptococcus agalactiae* (Lancefield's group B) isolated during the study of cow mastitis and 20 strains from excised tonsils and other human sources have been studied in detail and the results compared.

Methods. Carbohydrate and other media. Beef heart infusion broth (Difco) was used as a basic medium with Brom-cresol purple as indicator. The carbohydrate solutions were sterilized by filtration and then added to 5 cc of the sterile basic medium to give a final concentration of 1%. The sugar media were inoculated with one drop of an 18-hour growth of the streptococci in beef heart infusion broth. The aesculin medium was prepared according to Diernhofer[5] as cited by Plastridge *et al.*[6]

The methylene blue milk medium was prepared containing 1:5000 final dilution of the dye as recommended by Avery[7] and also containing the dye in 1:20,000 dilution. The hydrolysis of sodium hippurate was tested by growing the organisms in the original medium described by Ayers and Rupp[8] as well as in that recommended by Coffey and Foley.[9]

Grouping of streptococci. We used commercial grouping serums or antiserums prepared by us by immunizing rabbits with standard strains supplied by Dr. Lancefield. The extracts for the precipitin tests were made by Fuller's[10] method.

Results. The fermentation reactions of all the human and bovine cultures are given in Table I.

All the bovine organisms fermented lactose. Eight of the 20 human strains did not ferment this substance. All the human and bovine strains fermented dextrose, saccharose, maltose, levulose, trehalose and galactose. The great majority of the cultures fermented these substances overnight. All the human strains fermented dextrin, one bovine strain did not ferment this substance and 10 gave a doubtful reaction.

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* This work has been possible through a grant of the insular Department of Agriculture.

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[9] Coffey, J. M., and Folley, G. E., *Am. J. Pub. Health*, 1937, **27**, 927.

[10] Fuller, A. T., *Brit. J. Exp. Path.*, 1938, **19**, 130.

TABLE I.
Fermentation Reactions* of Lancefield's Group "B" Streptococci Obtained from Human and Bovine (Mastitis) Sources.

No. of Bovine strains / Human strains	Lactose	Dextrose	Saccharose	Maltose	Salicin.	Levulose	Trehalose	Glycerol	Dextrin	Galactose	Mannitol Xylose Dulcitol Rhamnose Adonitol Erythritol Sorbitol Arabinose Amigdalin
9/5	+	+	+	+	+	+	+	+	+	+	—
1/0	+	+	+	+	+	+	+	—	—	+	—
1/0	+	+	+	+	—	+	+	—	+	+	—
18/2	+	+	+	+	+	+	+	—	+	+	—
1/0	+	+	+	+	±	+	+	—	±	+	—
1/0	+	+	+	+	—	+	+	—	±	+	—
1/0	+	+	+	+	±	+	+	—	+	+	—
3/0	+	+	+	+	±	+	+	—	±	+	—
8/5	+	+	+	+	+	+	+	±	+	+	—
7/0	+	+	+	+	+	+	+	±	±	+	—
0/7	—	+	+	+	+	+	+	+	+	+	—
0/1	—	+	+	+	+	+	+	±	+	+	—

Total No. of human strains examined—20.
Total No. of bovine strains examined—50
* Observations were made daily during a period of two weeks.

TABLE II.
Fermentation of Glycerol by Lancefield's Group "B" Streptococci of Human and Bovine Origin.

No. and % of strains	Bovine +	Bovine ±	Bovine —	Human +	Human ±	Human —
No.	9	15	26	12	6	2
%	18	30	52	60	30	10

+, Positive; ±, Doubtful; —, Negative.
Period of observation—2 weeks.

The fermentation of glycerol is summarized in Table II.

Simmons and Keogh[4] reported that all their human strains fermented glycerol. They stated, however: "Fermentation of glycerol was not apparent until the third to the sixth day; a few strains did not ferment until later, up to the 14th day." We found also that the fermentation of glycerol was much delayed. The positive strains took from 6 to 13 days to ferment this substance.

All our human strains fermented salicin overnight. Two of the bovine strains failed to ferment this substance and 3 gave a doubtful reaction after 2 weeks.

No strains fermented mannitol, xylose, dulcitol, rhamnose, adonitol, erythritol, sorbitol, arabinose or amigdalin.

Twelve human strains showed the same fermentation reactions of one or more of the bovine cultures.

None of the bovine strains and only 2 human strains reduced methylene blue in 1:5000 dilution. Twenty bovine strains and all the human strains reduced this dye in 1:20,000 dilution.

All the bovine cultures and 18 of the 20 human cultures hydrolyzed sodium hippurate. No strains were able to split aesculin.

Virulence for mice. Fifty bovine and 20 human strains were tested for virulence. The organisms were grown in 5 cc of tryptose phosphate broth (Difco). The 18-hour growth was used for inoculation. All the mice used came from the same colony and weighed from 16 to 20 g approximately. Two animals were inoculated intraperitoneally with 0.5 cc and 0.1 cc respectively of each culture. After death the heart's blood was cultured and if streptococci were present they were grouped by Lancefield's method[11] utilizing Fuller extracts.[10]

Only 2 bovine strains (4%) killed mice when 0.5 cc of the culture was given intraperitoneally. In both cases group B streptococci were recovered from the heart's blood.

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[11] Lancefield, R. C., *J. Exp. Med.*, 1933, **57**, 571.

None of the animals receiving 0.1 cc of the culture died during the 10-day period of observation. Thirteen human strains (65%) killed mice when 0.5 cc of culture was injected intraperitoneally.

Of these 13 strains 3 also killed when 0.1 cc was inoculated. All the animals died in from 24 to 48 hours after inoculation.

Summary and conclusions. The biological properties and mouse virulence of 70 Lancefield's group B cultures (50 bovine and 20 human) have been studied and compared. The fermentation reactions show that, aside from the inability to ferment lactose by 8 of the human strains and some difference in the fermentation of glycerol, dextrin and salicin, the reactions are very similar for both groups. In some instances the biological properties studied were identical for strains of bovine and human origin. It has been observed with the majority of strains that the differences between single bovine and human cultures were not greater than those between individual strains from the same source.

There is an indication that human strains are more likely to reduce methylene blue.

It is known that the mouse virulence of group B streptococci is low. Inoculation of the 70 cultures studied into mice gave results which indicated that the strains from human sources possessed a higher mouse virulence than the bovine strains.

In spite of minor differences between cultures from bovine and human origin, their similarity in other respects points to a close relationship between these organisms.

15811

Latex Rubber Capsule for Producing Hypertension in Rats by Perinephritis.

MORRIS ABRAMS AND S. SOBIN. (Introduced by E. M. Landis.)

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In studies of dietary choices by hypertensive rats[1] it was necessary to produce large numbers of animals with graded and persistent hypertension. Constricting perinephritis was the method of choice for several reasons but the use of cellophane,[3,4] collodion[5] or silk[5,6] in young rats is technically difficult, the mortality is high, and the results are variable. Partial nephrectomy[2] could not be used because of the accompanying trauma and renal insufficiency. Simple compression of the renal artery had the disadvantage of technical difficulty coupled with later development of collateral circulation.

Enveloping the kidneys of young rats in molded pure gum latex capsules had previously been used to produce hypertension in a few animals in this laboratory.[7] This paper describes more complete experiments, indicating that this method can produce persistent hypertension ranging from 165 to 240 mm Hg at the end of 2 or 3 weeks in at least 70% of the total operated rats and in

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[1] Abrams, M., and Landis, E. M., to be published.

[2] Chanutin, A., and Ferris, E. B., Jr., *Arch. Int. Med.*, 1932, **49**, 767.

[3] Friedman, B., Jarman, J., and Klemperer, P., *Am. J. Med. Sci.*, 1941, **202**, 20.

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[5] Grollman, A., and Williams, J. R., Jr., *Am. J. Med. Sci.*, 1942, **204**, 73.

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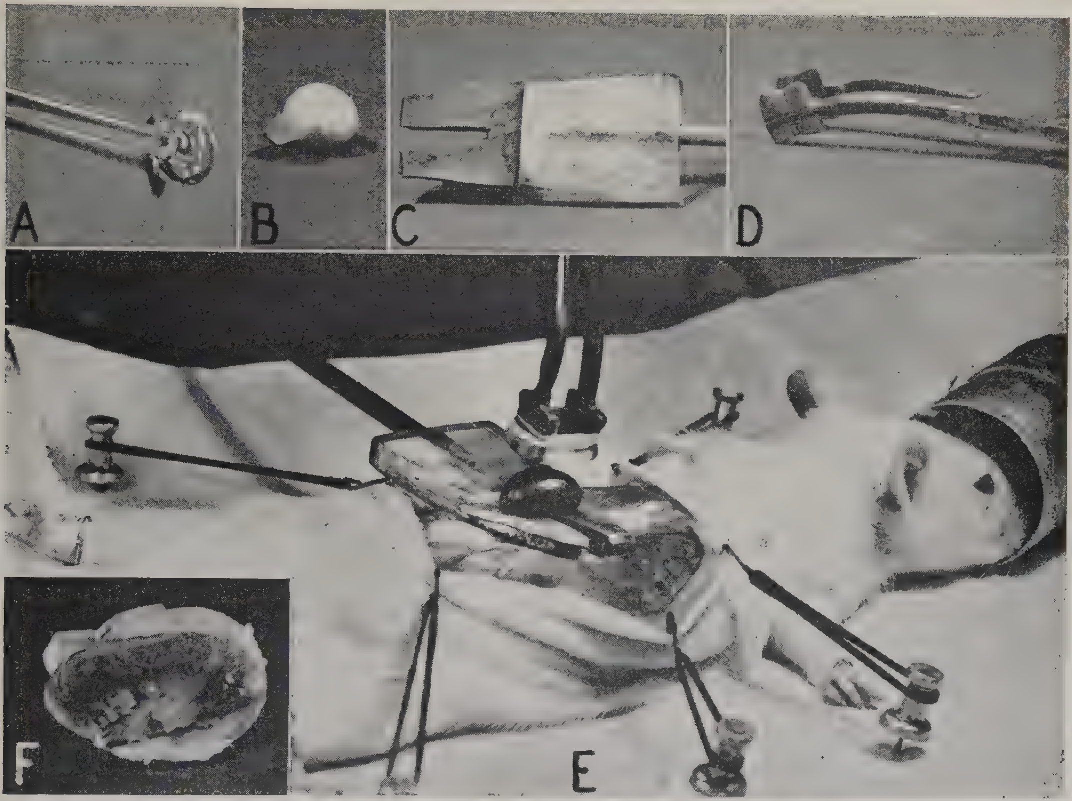

FIG. 1.

A. Glass mold.

B. Completed latex rubber capsule removed from mold.

C. V-shaped platform for stabilizing the delivered kidney while the capsule is being applied.

D. Kelly hemostat with ends broadened by means of solder to stretch and hold the everted capsule during application to kidney.

E. Operative field showing kidney in place on platform with stretched latex capsule held over the kidney prior to application.

F. Sagittal section of kidney showing thick, tough capsule 57 days after latex rubber capsule was applied. Rubber capsule itself has been removed completely.

about 90% of 60-day survivors. The convenience and dependability of the method, particularly when large numbers of rats with stable hypertension are needed, makes it worthy of brief description.

Method. A series of glass molds, resembling rats' kidneys in size and shape, were made by fusing and shaping solid glass rods or tubes (Fig. 1A). The molds were then dipped into liquid latex and placed in a rack, stem downward, to dry. Three dippings, producing a thickness of approximately 0.003 inch, plus a heavier reinforcing coat applied with camel's hair brush in the angle between the capsule and its neck made the rubber membrane suitably rugged and elastic. The completed capsules, still on the molds, were soaked in running hot tap water (45-50°C) for several hours to toughen the rubber, dissolve residual protein, and assist "peeling-off" the finished capsules (Fig. 1B). Assorted sizes of completed capsules were stored in 75% alcohol until used.

Male rats, weighing 190 to 200 g, were anesthetized with ether and fastened in the prone position (Fig. 1E). The hair over the lumbar region was removed and the skin scrubbed with 75% alcohol. Through a single dorsal mid-line incision, 5 to 6 cm long, either kidney could be delivered by the usual approach, *i.e.* by cutting the subcutaneous fascia longitudinally and splitting the under-

TABLE I.
Systolic Blood Pressures (Indirect) 60 Days After Applying Latex Rubber Capsules Bilaterally.

	First series	Second series	Totals, both series	%, both series together
Total No. operated	37	10	47	100
Deaths—1 to 30 days				
1 to 5 days post-op.	7	0	7	
6 to 30 days post-op.	4*	1	5	25.5
Survivors—30 days or more				
Systolic blood pressures†				
185 to 240 mm Hg	13‡	7	20	42.5
165 to 185 mm Hg	10§	2	12	25.5
135 to 165 (failures)	3	0	3	6.5

* Sacrificed 30 days after operation because of infected wounds and/or emaciation.

† Concurrent control rats, 11 in all, had systolic blood pressures ranging from 100 to 130 mm Hg.

‡ One died 57 days after operation in severe cardiac failure.

§ One died 42 days after operation with gross hematuria and acute urinary retention.

lying dorsal muscle mass by blunt dissection. In addition, splitting the muscles close to the vertebral column avoided undue stretching of the renal vessels during further manipulation of the kidney.

The upper and lower poles of the kidney were freed gently from the adrenal glands and perirenal fat by blunt dissection. Decapsulating the kidneys decreased the immediate postoperative mortality from renal insufficiency and was accomplished by nicking the capsule and peeling it toward the hilus with tissue forceps. Any oozing of blood was readily controlled by gentle pressure with a moist sponge.

The liberated and decapsulated kidney was then placed on the platform (Fig. 1C and E) with its pedicle in the V-shaped cleft. The soft rubber covering of this platform reduced trauma and prevented slippage. A rubber capsule of a size which would snugly enclose, but not compress, the kidney was rinsed free of all alcohol in warm Ringer's solution and placed in the everted position over the widened end of the hemostat used as an applicator (Fig. 1D and E). With the handles of the hemostat spread, the widely expanded rubber capsule was brought down over the kidney and the ends of the applicator pushed firmly against the kidney platform.

The lower free border of the everted capsule was drawn over the lower pole of the kidney by means of forceps, after which the other end of the capsule applicator was quickly turned inward, causing the upper end of the capsule, guided by forceps, to snap in place over the upper pole of the kidney. The loose neck of the capsule did not compress the renal vessels or ureter. The first kidney having been replaced, the other was treated similarly at the same operation and the rats were placed in individual metabolism cages for recovery. If oliguria or gross hematuria appeared during the night, 5 cc of Ringer's solution were given intraperitoneally and repeated again in 12 hours.

Sham operations, including all steps except the final snapping on of the latex capsule were performed in 5 animals. Systolic blood pressures in control, sham-operated and experimental rats were measured indirectly twice weekly by the plethysmographic technic described by Sobin.[7] All rats received Purina laboratory chow and water *ad lib.*

Results. Table I summarizes results obtained 60 days after bilateral application of latex rubber capsules in (a) an exploratory series of 37 male rats and (b) a more completely studied and technically superior series of 10 male rats. The highest normal systolic blood pressure in a parallel control series of 11 male rats was 130 mm Hg, the lowest 100 mm Hg. Because small changes in blood pressure are apt to be misleading, a rise of systolic blood pressure to

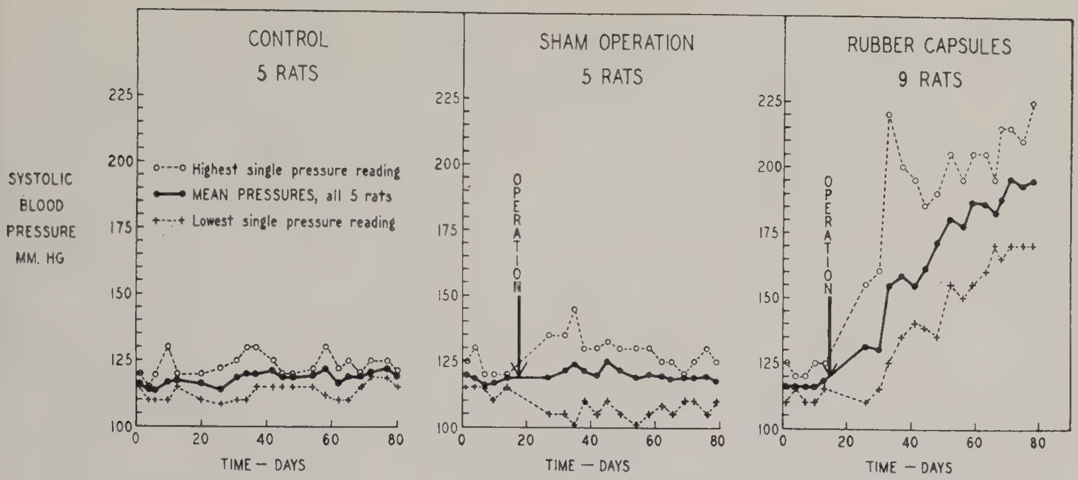

FIG. 2.

Charts showing systolic blood pressures of control rats, of sham-operated rats, and of rats with perinephritic hypertension due to bilateral application of rubber capsules.

TABLE II.

Comparison of Blood Pressures Measured (a) plethysmographically and (b) by Hamilton manometer.

	Indirect readings (plethysmograph on tail)	Direct readings (Hamilton manometer, femoral artery)	
	Systolic mm Hg	Systolic mm Hg	Diastolic mm Hg
Control rats	125-130	130-140	80- 85
	125-130	125-140	85- 90
	115-120	115-125	80- 90
Sham-operated rats (60+ days post-op.)	122-127	120-130	90- 95
	105-110	100-115	60- 70
	125-130	130-140	80- 90
Rats with bilateral latex rubber capsules (60+ days post-op.)	185-190	190-210	100-120
	205-210	190-220	110-130
	200-205	200-210	140-150

165 mm Hg or more was regarded as significant, *i.e.* any animal showing a rise less than 35 mm Hg above the highest single reading ever observed in parallel control animals was recorded as a failure.

The results for a total of 47 operated rats have been combined in Table I. As might be expected, experience led to improved results in the second series. In the first group mortality was 30%, in the second 10%. Similarly the incidence of significant hypertension was greater in the second and technically superior series. Severe hypertension (systolic blood pressures between 185 and 240 mm Hg) developed in 70% of the second series; moderate hypertension (165 to 185 mm Hg) in 20%.

Fig. 2 compares, for the second series, the highest single blood pressure, the lowest single blood pressure, and mean pressures for (a) 5 control rats, (b) 5 sham-operated rats, and (c) the 9 successfully operated rats all of which developed significant hypertension. The sham operation produced only a transitory increase in the systolic pressure of a few animals, while mean pressure for the group was not modified. Applying the latex capsules led to a clear increase in mean pressure by 2 to 3 weeks after operation and all of the animals were clearly developing hypertension by 3 weeks after operation.

Systolic blood pressures having been followed by repeated indirect measurements for 60 days, these conclusions as to the severity of the hypertension were verified by direct determinations using the Hamilton manometer. Three rats of each group were lightly anesthetized by injecting intraperitoneally 3 mg sodium pentobarbital per 100 g body weight. Their pressures were measured (a) plethysmographically and (b) immediately afterward directly from the femoral artery. Table II summarizes this comparison and, within the usual respiratory variations of blood pressure when a tracheal cannula is not used, shows the quantitative accuracy of conclusions drawn from previous indirect measurements.

Comment. The pathogenesis of hypertension in these animals appears to be similar to that produced by other chronically irritative materials such as silk or cellophane. Within a few days after the application of the rubber capsule the superficial tissues of the renal cortex reacted by forming first a fibrinous, then a definitely fibrous, envelope. The latter can become very thick and tough as shown in Fig. 1F. In this specimen the latex capsule has been applied to the kidney 57 days before. The microscopic changes were also similar to those produced by other types of irritative capsule. Similar results have also been observed in a few rabbits.

Summary. Enclosing both kidneys in molded latex rubber capsules to produce perinephritis can be recommended for its simplicity and effectiveness, particularly when large numbers of hypertensive rats are required.

15812

On Use of Urea- and Phenol-Treated Skin Test Antigens for Diagnosis of *Lymphogranuloma venereum.*

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It has been shown in an earlier report from this laboratory[1] that urea in concentrations of 1 to 2% inactivated the virus of *Lymphogranuloma venereum* in 10% yolk sac suspensions prepared from infected chick embryos. Suspensions so inactivated with urea were satisfactory both as complement-fixing and skin test antigens. Urea-inactivated skin test antigens (containing 0.25% phenol and 1-20,000 merthiolate as preservatives) have been prepared in these laboratories for clinical testing for over 4 years.[2-5]

When later studies[6,7] revealed that the addition of phenol to yolk sac suspensions enhanced their complement-fixing activity from a titer of approximately 1-200 to 1-3200, and in some instances to 1-6400, the question of the possible enhancement with phenol of the skin test activity naturally presented itself.

Methods. To investigate the effect of phenol on the reactivity of skin test antigens, saline suspensions were prepared as previously described[7] from yolk sac membranes heavi-

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[1] Nigg, C., PROC. SOC. EXP. BIOL. AND MED., **1942**, **49**, 132.

[2] Grace, A. W., Rake, G., and Shaffer, M. F., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 259.

[3] Blair, J. E., *J. Immunol.*, 1944, **49**, 63.

[4] Florman, A. L., *J. Immunol.*, 1945, **51**, 29.

[5] Dulaney, A. D., and Packer, H., *J. Immunol.*, 1947, **55**, 53.

[6] Nigg, C., and Bowser, B., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 192.

[7] Nigg, C., Hilleman, M. R., and Bowser, B., *J. Immunol.*, 1946, **53**, 259.

TABLE I.
Comparison of Urea- and Phenol-treated Skin Test Antigens.

Susp. 96 10% saline Susp.-YS membranes fractions:	Description			Complement fixation titer with standard positive serum pool	Skin reactions with antigens diluted: 1-500*		1-2000*		1-8000*	
	Inactivation 3 wks with:	Preservative added to diluted antigens Phenol	Merthiolate		Pt. weak reactors†	7 days	Pt. moderate reactors†	7 days	Pt. strong reactors†	7 days
A	Urea 2% 2-4°C	0.25%	1-20,000	1-200	Hun	30‡	Dea	30	DeL	49
					Bla	36	Jen	42	Ben	49
					Wil	4	DeV	36	Tho	48
B	Urea 2% 2-4°C	—	"	1-200	Hun	24	Dea	30	DeL	25
					Bla	25	Jen	49	Ben	36
					Wil	9	DeV	30	Tho	48
C	Phenol 0.5% 37°C	0.25%	"	1-3200	Hun	36	Dea	16	DeL	49
					Bla	49	Jen	104	Ben	64
					Wil	9	DeV	56	Tho	48

* Dilutions are expressed in terms of original yolk sac weight.

‡ Score equals product of the axes of the erythematous papule in mm.

† Patients used in testing the three dilutions were selected on the basis of previous tests.

ly infected with the virus of *Lymphogranuloma venereum* and fractions thereof were treated as shown in Table I. Fraction A was prepared by the method in use in these laboratories for the routine production of *Lymphogranuloma venereum* skin testing antigens. Fraction B was prepared in identical manner. Fraction C was treated at 37°C for 3 weeks with phenol in a final concentration of 0.5%. This procedure effected enhancement of complement-fixing activity.[7] The final dilutions of Fractions A and C contained 0.5% phenol and 1-20,000 merthiolate as preservatives, while Fraction B contained only merthiolate. Control material for each fraction was prepared in identical manner from normal yolk sac membranes (in approximately the same stage of development) for simultaneous testing.

When all preparations had passed the necessary tests for sterility and inactivation of virus, they were tested on clinically and serologically positive cases of *Lymphogranuloma venereum,* using the 1-500 dilutions of each preparation on each of several patients, the 1-2000 dilutions on each of another group of patients and the 1-8000 dilutions on each of a third group. Such a procedure was followed to rule out differences in individual response to the same test material. The test dose of each preparation was 0.1 ml injected intracutaneously on the flexor surface of the forearm. The reactions were recorded at 2- and 7-day intervals following injection.

Results. Some patients show, during the first 2 or 3 days following the test injection, a more or less extensive edematous, erythematous area with or without induration. The erythema may recede fairly rapidly, leaving a characteristic erythematous papule which regresses slowly with or without central necrosis, requiring several weeks for its complete disappearance. This more persistent reaction, as seen one week after injection, was thought to present a more reliable criterion for the comparative evaluation of the various preparations. Table I, therefore, shows only the 7-day readings. The product of the axes of the erythematous papule in mm was used as a basis for scoring the reactions. In no case did the control Fractions A, B and C, give a positive reaction with 1-500 dilution. The results present at the most only slight and probably insignificant differences in reactivity between the 3 preparations, whether phenol was used in the preparations (Fractions A and C) or was completely absent (Fraction B).

The skin test results are in contrast to the comparative complement-fixing activities of Fractions A and B with a titer of only 1-200, and Fraction C, with a titer of 1-3200, constituting a 16-fold enhancement.

Summary. 1. Saline suspensions prepared from yolk sacs heavily infected with the virus of *Lymphogranuloma venereum* and inactivated with 2% urea or 0.5% phenol constituted satisfactory diagnostic skin test antigens in high dilutions.

2. Yolk sac suspensions treated with phenol so as to enhance their complement-fixing activity 16-fold were not appreciably more active as skin test antigens than urea-treated suspensions.

15813 P

Trypanocidal and Spirochetocidal Compounds Derived from BAL and Organic Arsenicals.

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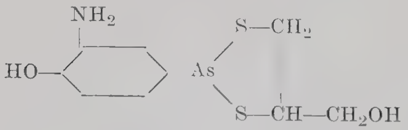
This study is part of an investigation of the chemotherapeutic activity of organometallic compounds as affected by chemical combination with dithiols.

War research has brought out the fact that BAL (British Anti Lewisite, 2,3-dimercaptopropanol) protects man and experimental animals against the toxic effect of organic arsenicals, including 4-arsenoso-2-aminophenol (I), *i.e.*, oxophenarsine U.S.P., and counteracts *in vitro* the trypanocidal effect of this drug.[1-3]

The limited information published to date (February 18, 1947) suggests that the compound resulting from the condensation of I with BAL, *i.e.*, 2-amino-4-[methylol-(ethylenedimercaptoarsino)]-phenol (II) would be significantly less toxic than its parent substance (I), but devoid of chemotherapeutic activity.



(II)

With a view to evaluating this proposition, we have synthesized (II) and tested *in vivo* its trypanocidal and spirochetocidal activity.

(II) crystallizes from methanol as white needles, soluble in propylene glycol and dilute hydrochloric acid, insoluble in water and anhydrous acetone. It gives a negative nitroprusside reaction at pH 8, positive at 10.

Analysis:

Calcd. for $C_9H_{12}O_2NS_2As$: N 4.59, As 24.6

Found: N 4.52, As 24.0

4.49 24.8

(II) forms a crystalline hydrochloride (III), soluble in water, ethanol and propylene glycol, insoluble in anhydrous acetone.

Analysis:

Calcd. for $C_9H_{13}O_2NClS_2As$: N 4.11; As 22.0

Found: N 4.10; As 21.2

The free base (II), and more so its hydrochloride (III), are quite stable. Propylene glycol solutions of the hydrochloride may be sterilized by heating for one hour at 100°.

(III) cures the experimental *T. equiperdum*

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[1] Peters, R. A., *Nature*, November 24, 1945.

[2] Waters, L. A., and Stock, C., *Science*, 1945, **102**, 601.

[3] Sulzberger, M. B., and Baer, R. L., *J. Am. Med. Assn.*, 1947, **133**, 293.

infection of the mouse with a single intraperitoneal dose of 0.02 g/kg, while the maximum tolerated dose, intraperitoneally, was found to be 0.12 g/kg, corresponding to a therapeutic index of 6. The immediate spirochetocidal effect of (II) was screened in rabbit syphilis: 16 animals with fully developed, dark-field positive chancres were treated with a single subcutaneous injection of (II) dissolved in propylene glycol in doses ranging from 2.5-40 mg (.0008-.013 g/kg). In all animals treated with doses equal to or greater than .006/kg the spirochetes were not demonstrable 5 days after the treatment. As to toxicity, a single intramuscular dose of .05 g/kg is well tolerated while .08 g/kg is lethal.

It follows from these experiments that the condensation product of a chemotherapeutically active arsenical with BAL may result in a new compound combining relatively low toxicity with a significant trypanocidal and spirochetocidal activity.

Similar results, to be reported in a forthcoming publication, have been obtained with analogous BAL derivatives of other aromatic arsenicals as well as with aromatic antimony compounds, including arsanilic acid, tryparsamide, carbarsone, stibanilic and acyl stibanilic acids.

Hence, in a general manner, inclusion of a phenyl substituted arsenic or antimony residue in a 5-membered sulfur-containing ring as in (II) is not incompatible with a significant chemotherapeutic activity.

The new BAL compounds seem of more than theoretical interest in view of their stability and solubility properties. As a group they are more soluble in nonaqueous solvents than the parent organometallics, a fact which may well have repercussions on their chemotherapeutic spectrum. The solubility, and consequently, the distribution within the organism, may readily be modified by replacing the BAL radical in the above-mentioned compounds by other dithiols, such as 2,3-dimercaptopropionic acid, BAL ethers, BAL glucoside, 1,2-dimercaptobenzene, etc.

The mechanism of the therapeutic effect of the BAL derivatives remains to be elucidated. There are at least 2 possibilities: (1) the molecule may act as a whole, which would imply a mechanism not foreseen by the "Ehrlich-Voegtlin SH-arsenoreceptor theory," (2) the BAL compounds are dissociated in the organism and act essentially like the parent compounds.

15814

Epithelial Keratinization as Evidence of Fetal Vitamin A Deficiency.*

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The appearance of stratified keratinized epithelium in places where such epithelium is not ordinarily found is the cardinal sign of vitamin A deficiency. It has been stated that this histologic change, referred to as keratinizing metaplasia, occurs in all vertebrates regardless of age.[1] However, it has not been described in fetal or newborn animals whose mothers were in a state of vitamin A deficiency during pregnancy. This is noteworthy, since Hale,[2] using the pig, and Warkany and Schraffenberger,[3] using the rat, obtained fetuses that bore severe morphologic

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* This work was aided in part by a grant from the Nutrition Foundation, Inc., N. Y.

[1] Wolbach, S. B., and Bessey, O. A., *Physiol. Rev.*, 1942, **22**, 233.

[2] Hale, F., *Am. J. Ophth.*, 1935, **18**, 1087.

[3] Warkany, J., and Schraffenberger, E., *Arch. Ophth.*, 1946, **35**, 150.

anomalies when the mothers were deprived of vitamin A during pregnancy.

The present communication is a report of the finding of keratinizing metaplasia in the genito-urinary tract of fetal rats taken from mothers that were deficient in vitamin A during pregnancy. It is based on a study of serial sections of 14 fetuses and newborns from as many different litters, the gestational age of which ranged from the 16th through the 22nd day. Maternal deficiency was induced and maintained by the same diets and experimental procedures as were used by Warkany and Schraffenberger.[3] Fetuses of comparable ages and newborn rats from mothers fed an adequate diet were sectioned and studied as controls in order to rule out the possibility of keratinization of genito-urinary epithelia by maternal estrogens during the latter part of pregnancy. The histology of the normal epithelia will not be described in this paper, and only those histologic features which represent distinct deviations from the normal will be described for the A-deficient animals. Nevertheless, it should be stated that keratinization normally does not occur in any part of the genito-urinary tract of fetal and newborn rats except in the most distal portion of the urethra, *i.e.*, the external orifice and a short segment contiguous with it.

Neither the control fetuses nor those subjected to maternal vitamin A deficiency exhibited keratinization in any part of the body prior to the 18th day of gestation. All fetuses older than 18 days whose mothers were fed the A-deficient diet, however, showed keratinizing metaplasia in at least some of the genito-urinary epithelia which constituted, or were derived from, the urogenital sinus. A precise localization of the affected epithelia in terms of definitive organs is not feasible in the present report, since the differentiation of the entire urogenital sinus was conspicuously retarded and this retardation, in turn, complicated by the occurrence of several frank malformations. Until a larger series of animals has been studied and plastic reconstructions prepared, no attempt will be made to analyze the malformations, and the location of areas of metaplasia will be described only in a general way.

The 18th day of gestation was the earliest time at which metaplastic keratinization appeared in the offspring of A-deficient mothers. A male fetus of this age was found to possess a small area of truly keratinized epithelium within the urethral plate and another area in the pelvic part of the urogenital sinus where, although no completely keratinized cells were seen, the cytoplasm of superficial cells was hyalinized and contained keratohyalin granules. These changes might be designated "prekeratinization." The findings seem particularly interesting in view of the fact that the epidermis and other ultimately cornified epithelia had not undergone keratinization in either this animal or control fetuses of the same age.

True keratinization, typified by cornification and desquamation of the surface-cells and by hyalinization and keratohyalin granulation in the superficial layers of cells, was consistently found in fetuses of a gestational age of 20 days. It was limited, however, to the distal two-thirds of the urogenital sinus, specifically to the segment lying between the point of juncture of the genital ducts with the sinus, cephalically, and the exterior of the body, caudally. Metaplasia occurred as a continuous strip along the dorsal wall of this segment but, curiously, had affected to a much less striking degree, or not at all, the lateral and ventral walls of this region of the sinus. The point at which keratinization was most pronounced, both in degree and in circumferential extent, was where the genital ducts joined the sinus. This was also the most cephalic part of the affected area. This fact speaks against the possibility that keratinizing changes spread into and along the sinus from the already keratinized epidermis on the exterior, and seems to indicate instead that an *in situ* transformation to a keratinized type of epithelium had occurred. No true cornification was seen in 20-day fetuses above the point of entry of the genital ducts, but prekeratinization changes, beginning hyalinization of the cytoplasm and the appearance of keratohyalin granules in the

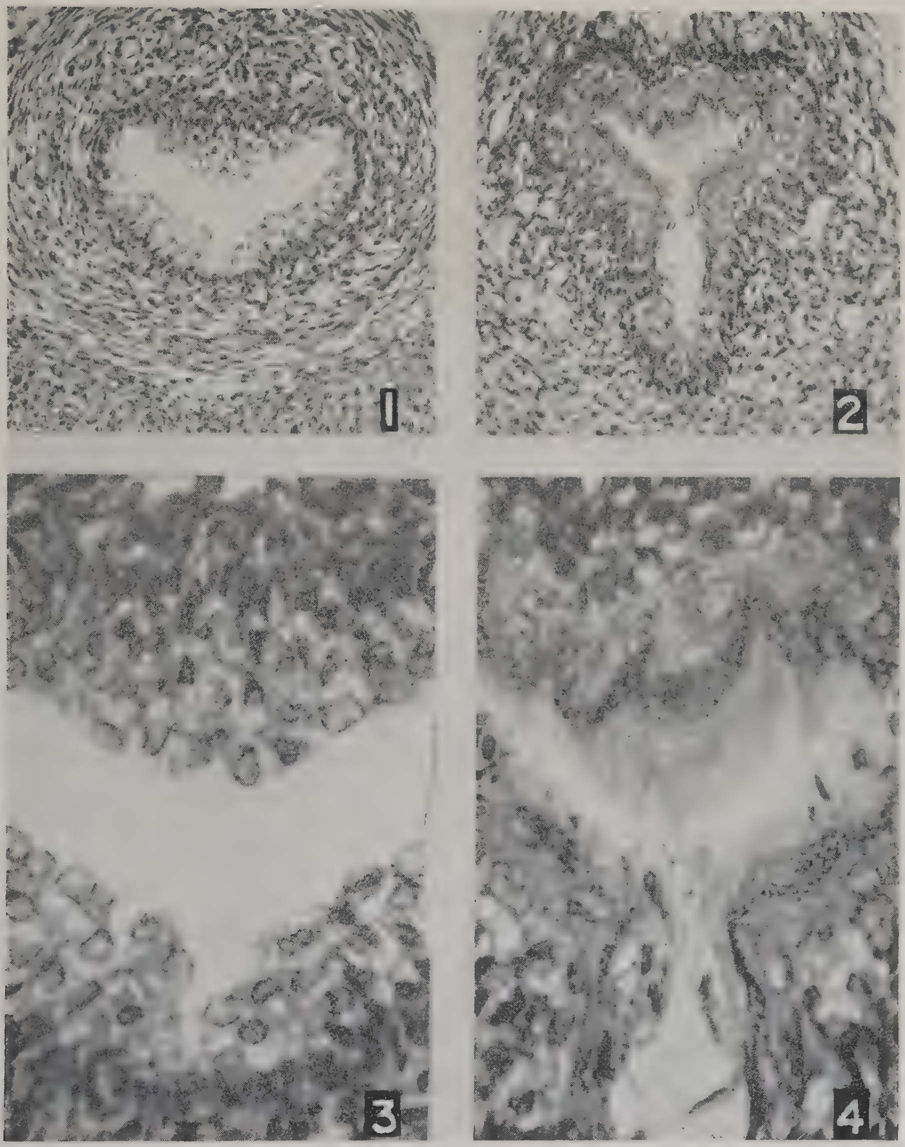

Fig. 1-4.

Fig. 1. Urethra at the level of the urethral sinus of a newborn rat, offspring of a mother fed an adequate diet. (×210)

Fig. 2. Urethra at the level of the urethral sinus of a newborn rat, offspring of a mother fed a vitamin A-deficient diet. (×210)

Fig. 3. Higher magnification of the urethra shown in Fig. 1. (×650)

Fig. 4. Higher magnification of the urethra shown in Fig. 2. (×650)

These photomicrographs are oriented with the "dorsal" wall toward the top of the page. As used here, the term "dorsal" refers to that wall of the urethra derived from the dorsal wall of the embryonic urogenital sinus, and does not accurately describe the relative position of this wall in the newborn rat.

surface-cells, were observed in the region of the upper sinus that roughly corresponded to the neck of the bladder.

Fetuses taken on the 21st and newborns taken on the 22nd day showed perhaps a more advanced degree of cornification (Fig. 1-4), with considerable desquamation at some points, but the extent of the affected area was scarcely greater than at 20 days. Again, structures derived from the urogenital sinus distal to its juncture with the genital ducts were keratinized throughout their length; and metaplasia was always more pronounced on, and tended to be limited to, the dorsal aspect of these structures. In one newborn male an area of true metaplasia was observed in the vesico-urethral portion of the sinus at a point corresponding to the upper prostatic urethra. Otherwise, only prekeratinization changes were seen in this more cephalic portion of the sinus. Even in these newborns and more mature fetuses no histologic deviations from normal were seen in the epithelia in regions of the definitive bladder, ureters, kidneys, mesonephric ducts or Müllerian ducts.

Comment. These observations reveal that genito-urinary epithelia in fetal rats, like many other epithelia in older animals, require vitamin A for their morphologic integrity. It appears that this requirement is not manifested by keratinizing metaplasia until the fetal tissues have undergone a certain degree of differentiation. In the urogenital sinus this is attained by, or shortly before, the 18th day of gestation. The fact that keratinization did not affect other epithelia, which in older animals do respond to A-deficiency by undergoing metaplastic changes, does not prove that the unaffected epithelia were differentiated to a lesser degree than those of the urogenital sinus. Nevertheless, it is suggestive in this direction, and it undoubtedly reflects some unobserved morphologic or perhaps functional difference between those tissues that undergo metaplasia during the fetal period and those that react in this fashion to A-deficiency only at an older age.

The prevalence of congenital malformations in the genito-urinary tract of these fetuses is probably somehow related to the particular susceptibility of epithelia in this region to keratinizing metaplasia, although any attempt to evaluate such a relationship must await a more thorough study of the malformations.

Summary. Keratinizing metaplasia in epithelia of the genito-urinary tract was observed in fetal rats taken from mothers fed a vitamin A-deficient ration. Only structures derived from the fetal urogenital sinus were affected. The earliest metaplastic changes were seen in a fetus of 18 days gestational age; by the 20th day keratinization was seen in the dorsal wall of all organs distal to the termination of the genital ducts. Epithelia in other parts of the body were not affected by these changes.

15815 P

Immunization of Mice with Dysentery Antigen Administered by Gavage and by Voluntary Drinking.*

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I. *Living and Killed Culture by Gavage.* In the course of attempts to infect white mice by gavage with living *Shigella sonnei,* we have observed the development of immunity. A high degree of immunity follows administration of large numbers of bacteria. Less immunity follows administration of fewer bacteria.

Fifteen mice were given 6 consecutive daily doses of 49 billion living bacteria with a syringe and blunt needle introduced into the stomach by way of the esophagus. Five days after the last dose, small groups of these mice were challenged intraperitoneally with 16 to

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* This study was conducted under a grant-in-aid from the U. S. Public Health Service.

TABLE I.
Immunity in Mice Given Living and Killed *Shigella sonnei* by Gavage. Challenged Intraperitoneally with 0.5 to 1280 MLD of Homologous Culture.

Type of antigen	% of mice surviving No. of doses of antigen					
	3	4	5	6	7	8
Living culture	57*	55	72	89	73	92
Killed ’’	70	50	71	76	85	83

* Each % is based on results with 30 mice.

TABLE II.
Immunity in Mice Drinking Killed *Shigella sonnei* in Place of Water for 21 Days.

No. of mice	Fluid consumed	Homologous challenge intraper. MLD	No. of mice surviving
36	Killed culture*	2 to 2048	36
30	Sterile broth	’’	2
31	Water	’’	2
36	’’†	’’	0

* 7.5 billion per day per mouse.
† Virulence control.

160,000 MLD of homologous culture suspended in sterile mucin.[1] All mice survived except one which received 160 MLD.

Smaller numbers of living or heat-killed bacteria were compared for their ability to stimulate active immunity when administered by gavage. Six groups of 30 mice were given 3 to 8 daily doses of 1.4 billion living *Shigella sonnei.* Six other groups of 30 mice were given the same number of doses of 1.1 billion killed organisms. Seven days after the last doses each group of 30 mice was divided into 6 equal groups and challenged intraperitoneally with 0.5 to 1280 MLD of homologous culture suspended in mucin. The percentages of mice surviving are found in Table I. The striking finding was that both living and killed culture yielded similar immunity which increased with increasing number of doses.

The majority of mice remained well during the time they were receiving living culture intragastrically. During this period and for 24 to 48 hours subsequently stool cultures were positive.

II. *Voluntary Drinking of Killed Culture in Place of Water.* A group of 36 white mice were given killed broth culture of *Shigella sonnei* in their water bottles in place of water over a period of 21 days. Another group of 30 mice were given sterile broth in place of water for the same length of time. A third group of 31 mice received only water to drink. Four days after the 21-day period each group of mice was challenged intraperitoneally with 2 to 2084 MLD of homologous culture in mucin. Data in Table II show that all mice survived which had ingested killed culture in place of water; that only 2 mice survived which had consumed sterile broth; and only 2 mice survived which had consumed water. The quantity of killed culture, sterile broth and water consumed daily by each mouse was very similar.

Conclusions. Six daily doses of 49 billion living *Shigella sonnei* administered by gavage to white mice stimulated complete immunity to intraperitoneal injection of 16 to 160,000 MLD of homologous culture suspended in sterile mucin. Killed bacteria in doses of 1.1 billion and living bacteria in doses of 1.4 billion stimulated immunity, the titer of which increased with the number of daily doses. There was no significant difference in the immunizing effects of living and killed culture. Mice which drank killed broth culture in place of water for 21 days were immune against 2 to 2048 MLD of homologous culture injected intraperitoneally.

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[1] Cooper, Merlin L., and Keller, Helen M., *J. Pediat.*, 1941, **18**, 451.

15816 P

Effect of Adrenalectomy upon Survival Time of the Eviscerated Rat.

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This is one of a series of studies of endocrine deficiency and excess in the eviscerated rat. It was shown that removal of the adrenal glands shortens the survival of the eviscerated rat under several experimental conditions.

Methods. Male rats of the Sprague-Dawley strain were fed Purina Dog Chow. At a weight of 185 to 205 g, the inferior vena cava was ligated between the liver and kidneys to cause the development of a collateral circulation. Asepsis was preserved. When the animals reached 250 ± 2 g they were anesthetized (intraperitoneal injection of 18 mg of cyclopentenyl-allyl-barbituric acid sodium) and eviscerated.[1] The vascular supply of the adrenals and the kidneys was ligated and these organs removed at the time of evisceration.

The temperature of the room was 75° to 78°F and the humidity 30 to 35% of saturation. Crystalline zinc insulin (Lilly) was given to some of the animals (Exp. 3 and 4). It was injected into the saphenous vein of the right hind leg with an initial dose of 4 units and a subsequent steady rate of 40 units per 24 hours by means of a continuous injection apparatus. Injections were started within 2 minutes following removal of the liver. Cessation of heart beat was the criterion used to judge the time of death.

Experiments and Results. Ten adrenalectomized and 10 nonadrenalectomized rats were studied in each experiment. Comparisons of adrenalectomized and nonadrenalectomized animals were always made simultaneously on pairs.

In Exp. 1 (kidneys intact, no insulin) the nonadrenalectomized animals survived an average of 870 minutes with a range of 535 to 1467, and the adrenalectomized an average of 361 minutes with a range of 125 to 724 minutes.

In Exp. 2 (nephrectomized, no insulin) the nonadrenalectomized animals survived an average of 183 minutes with a range of 152 to 222, and the adrenalectomized an average of 139 minutes with a range of 67 to 175.

In Exp. 3 (kidneys intact, insulin) the nonadrenalectomized rats survived an average of 86 minutes with a range of 65 to 123, and the adrenalectomized an average of 72 minutes with a range of 57 to 99.

In Exp. 4 (nephrectomized, insulin) the nonadrenalectomized rats had an average survival of 76 minutes with a range of 64 to 85, and the adrenalectomized an average of 61 minutes with a range of 43 to 70 minutes.

In 39 of the 40 pairs of rats studied in these experiments the adrenalectomized animal survived a shorter period than its nonadrenalectomized control.

Discussion. The study of the effect of hormone deficiency and excess upon short survival times may, as in the present experiment, provide one index of the time required for hormonal effect.

It was previously known that either nephrectomy[2] or the administration of insulin[3] hastens the onset of hypoglycemia and death of the eviscerated rat. In these experiments it was found that the survival time of the eviscerated-nephrectomized rat is decreased still further by the injection of insulin. All of the animals dying under these conditions show marked hypoglycemia, and die with convulsions and respiratory failure. In each case the symptoms can be prevented and survival greatly prolonged by the administration of glucose.

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[1] Ingle, D. J., and Griffith, J. Q., Chapter 16, *The Rat in Laboratory Investigation*, J. B. Lippincott Co., Philadelphia, 1942.

[2] Reinecke, R. M., *Am. J. Physiol.*, 1942, **136**, 167.

[3] Ingle, D. J., and Sheppard, R., *Endocrinology*, 1945, **37**, 377.

The presence or absence of the adrenal glands modifies the survival of the eviscerated and the eviscerated-nephrectomized rat either with or without insulin. This effect of adrenalectomy can be observed within 2 hours following operation. The data do not permit a differentiation between the relative importance of the adrenal medulla and the adrenal cortex in effecting the survival of the eviscerated rat.

Summary. The survival of the eviscerated rat is shortened by either nephrectomy or insulin. Insulin shortens the survival of the eviscerated-nephrectomized rat. Adrenalectomy at the time of evisceration shortens the survival of the eviscerated and eviscerated-nephrectomized rat either with or without insulin.

15817

Enhancement and Depression of Neuromuscular Transmission by Intra-arterial Injection of Potassium Chloride in the Dog.

Sheppard M. Walker. (Introduced by A. S. Gilson, Jr.)

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Rosenblueth and Cannon[1] observed that intra-arterial injection of KCl, during indirect stimulation of muscle in the cat at a high frequency, produced marked depression of muscle tension. More recent studies[2,3] have shown that intraperitoneal injection of KCl improved neuromuscular transmission in the rat and that intra-arterial injection of KCl in the nerve-muscle preparation from the frog first augmented and then depressed neuromuscular transmission. The present paper is concerned with the effect of varied rates of intra-arterial injection of KCl on the development and maintenance of muscle tension during stimulation of the nerve at a high frequency. Nine dogs anesthetized with sodium barbital were used in the experiments. Both *Triceps surae* were prepared as previously described for the rat.[2] Spring levels were used for the recording of muscle tension. The muscles of each leg were stimulated simultaneously through the cut sciatic nerves with electrodes connected in parallel and led from a Thyratron stimulator. The stimulus strength was about 6 times threshold and the frequency of stimulation was 275 to 300 shocks per second. The duration of stimulation was usually 5 or 10 seconds with an equal interval of rest between periods of stimulation. In some experiments stimulation was continuous during the period of injection. In a few experiments the muscles were stimulated with single shocks during the period of injection. Injections were made into the femoral artery. Two rates of injection were employed: slow, 2 to 3 mg of KCl (1.12% solution) per second; fast, 15 mg of KCl (4.2% solution) per second.

Results. During indirect stimulation at 300 shocks per second the peak of tetanic tension was followed immediately by a rapid decline of tension to a much lower level which was either maintained or slightly increased during continued stimulation (Fig. 1, 1A). Slow injection of KCl gradually reduced the rate and the extent of decline of tension in the perfused muscles during successive periods of stimulation. The maximal enhancement of tetanic tension was attained approximately one minute after the beginning of injection (Fig. 1, 1B). In the contralateral muscles, not subjected to KCl perfusion, little change in the tension curve occurred during the same interval (cf. 2A and 2B of Fig. 1). By the end of 2 minutes of slow injection

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[1] Rosenblueth, A., and Cannon, W. B., *Am. J. Physiol.*, 1940, **130**, 205.

[2] Walker, S. M., *Am. J. Physiol.*, 1947, **149**, 7.

[3] Walker, S. M., and Laporte, Y., *J. Neurophysiol.*, 1947, **10**, 79.

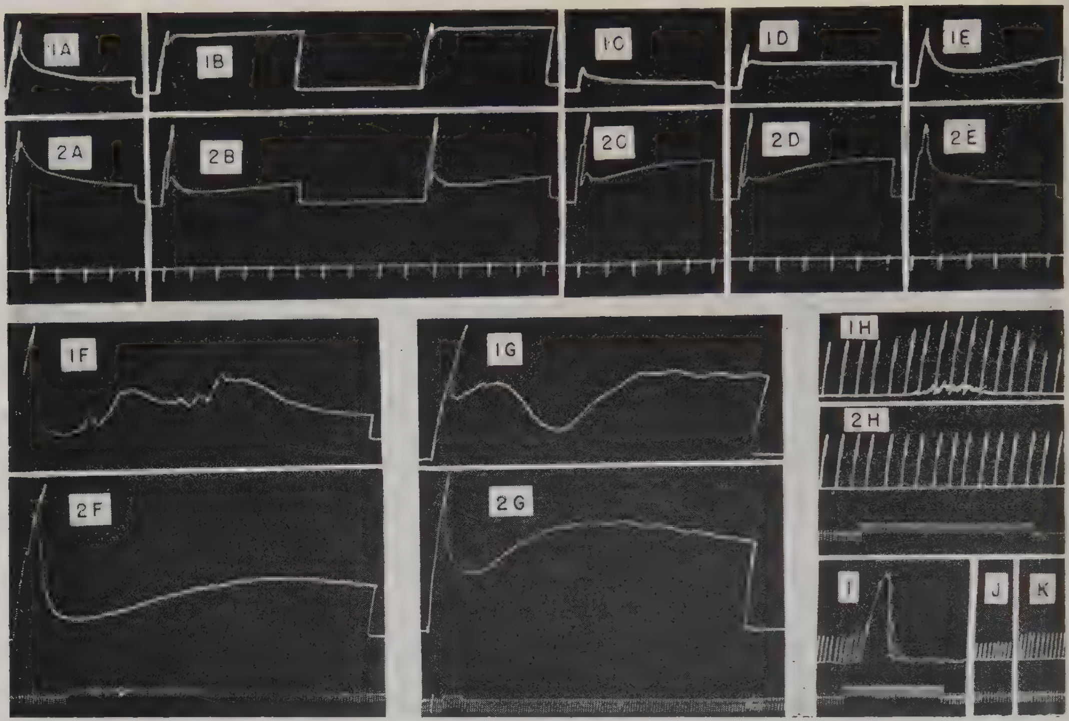

Fig. 1. Myograms showing the effect of intra-arterial injection of KCl on the response of the *Triceps surae* to electric stimulation of the cut sciatic nerve. Time signal: 1-second intervals. Elevations of the time line show periods of injection.

1A to 1E: Recordings of tension from muscles perfused with 2 mg of KCl (1.12% solution) per second for 2 minutes and stimulated during alternate 5-second intervals with 300 shocks per second. 1A: Before injection. 1B: After 1 minute of injection. 1C: After 2 minutes of injection. 1D: One minute after injection was stopped. 1E: Ten minutes after injection was stopped. 2A to 2E: Recordings of tension from the contralateral muscles stimulated in parallel with the muscles recorded in 1A to 1E.

1F: Muscles perfused with 3 mg of KCl (1.12% solution) per second for 54 seconds during continuous stimulation at 275 shocks per second. 2F: The contralateral muscle stimulated in parallel.

1G: Muscles perfused with 15 mg of KCl (4.2% solution) per second for 7 seconds during continuous stimulation at 275 shocks per second. 2G: The contralateral muscles stimulated in parallel.

1H: Muscles perfused with 3 mg of KCl (1.12% solution) per second for 68 seconds during stimulation at 1 shock per 5 seconds. 2H: The contralateral muscles stimulated in parallel.

I: Muscles perfused with 15 mg of KCl (4.2% solution) per second for 40 seconds during stimulation at 1 shock per 1.5 seconds. J: The same muscles recorded in I showing partial recovery of neuromuscular transmission 20 minutes after the injection. K: The same muscles recorded in I showing complete recovery of transmission 25 minutes after the injection.

the perfused muscles showed marked depression of tetanic tension, while the response of the contralateral muscles showed only slight change (Fig. 1, 1C and 2C). At this point injection was discontinued. An increase of tetanic tension occurred in each successive period of stimulation of the muscles which had received intra-arterial injection of KCl (Fig. 1, 1D). Ten minutes after injection was stopped the tension curve was almost normal (Fig. 1, 1E).

Slow injections made during continuous stimulation at 275 shocks per second produced an increase of tension followed by a decline (Fig. 1, 1F). If injections were stopped shortly after the decline began another rise of tension occurred. If, on the other hand, injections were continued for several seconds after the decline appeared no increase of tension occurred after cessation of injection. When fast injections were given during stimulation a depression of tension began a few seconds after the start of injection. If the duration of injections was only 6 to 8 seconds an increase of tension followed shortly after the cessation of injection (Fig. 1, 1G). Longer periods of injection produced either a prolonged depression of tension or complete neuromuscular block.

The typical potentiation of muscle response to single shocks was produced by slow injections (Fig. 1, 1H). It is of interest to note that continuation of slow injection induced slight contracture which was followed shortly by diminution of response to single shocks. When injection was stopped at the onset of depression recovery of the normal response occurred within 10 to 20 seconds. Marked contracture and complete neuromuscular block were produced by prolonged fast injections (Fig. 1I). Twenty to 25 minutes were required for recovery of the response to single indirect stimuli (Fig. 1, J and K). It was shown that the quantity of KCl required to produce complete neuromuscular block did not impair the contractile strength of the muscle stimulated directly with single or with high-frequency shocks.

Discussion. Direct stimulation in the curarized muscle of the rat[4] has shown that KCl-treatment does not increase tetanic tension. Therefore, it is assumed that all enhancement of tetanic tension observed in this study, except increases resulting from contracture, is due to improvement of neuromuscular transmission. The presence of contracture usually could be recognized by irregularities in the tension curve (Fig. 1, 1F). The slowest rate of injection of KCl (2 mg per second) produced no contracture although marked augmentation of tetanic tension occurred (cf. 1A and 1B of Fig. 1). Moreover, it seems likely that the contracture during injection of 3 mg per second of KCl produced only a small portion of the increased tension observed at a high frequency of stimulation (cf. the slight increase of tension induced by contracture in 1H with the marked increase of tension during KCl injection in 1F).

The observation that neuromuscular transmission is facilitated by intra-arterial injection of small quantities of KCl confirmed the results obtained by intraperitoneal injection of KCl in the rat.[2] Furthermore, the failure of the injection to induce similar changes in the contralateral muscles showed that the results were not attributable to central effects on the circulation or respiration. The observations of enhancement of neuromuscular transmission with small amounts of KCl and of depression with large amounts are in agreement with the findings in the curarized nerve-muscle preparation from the frog.[3] Moreover, the present results in the dog have shown that both the augmenting effect and the depressant action of K on neuromuscular transmission occur in uncurarized muscle.

Summary. 1. Continuous slow injection of KCl (2 to 3 mg per second of 1.12% solution) produced initial enhancement and subsequent depression of neuromuscular transmission in muscle stimulated indirectly at 275 to 300 shocks per second.

2. Fast injection of KCl (15 mg per second of 4.2% solution) produced a prompt depression of neuromuscular transmission. Continued injection produced marked contracture and complete neuromuscular block.

3. Enhancement of neuromuscular transmission may occur in the absence of contracture or in the presence of mild contracture. Amounts of K sufficient to induce marked contracture usually produced complete neuromuscular block.

4. During complete neuromuscular block by KCl injection the response of the muscle to direct stimulation was unimpaired.

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[4] Walker, S. M., unpublished data.

15818

Further Studies on the Acute Toxicity of Mercurial Diuretics.*

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A number of reports have appeared concerning a serious and sometimes fatal reaction which has been observed following intravenous injection of therapeutic doses of mercurial diuretics.[1-3] This has been shown to be due to a direct action on the heart giving rise to a variety of changes in rhythm and conduction[4,5] and a decrease in work capacity.[6] The reaction appears to be characteristic of mercurials in general, whether organic or inorganic, and not specifically of the diuretic group.

In a previous report, the acute lethal dose was given for the common diuretics as estimated by intravenous injection in intact unanesthetized cats. This work has now been extended to determine the comparative dosages at which cardiac toxicity develops as indicated in the electrocardiogram. The drugs used in the present comparisons were:

Mercuric Chloride: A 5.4% solution in distilled water.

Mercuzanthin: A solution of the sodium salt of N(γ-hydroxymercuri-β-methoxy) propyl camphoramic acid-theophylline (Mercurophylline Injection U.S.P. XIII).

Salyrgan-Theophylline: A solution of the sodium salt of ortho-N(γ-hydroxymercuri-β-methoxy)propyl carbamyl phenoxy acetic acid-theophylline (Mersalyl and Theophylline Injection, U.S.P. XIII).

Mercuhydrin: A solution of the sodium salt of N(γ-hydroxymercuri-β-methoxy) propyl N′(succinyl)urea and theophylline.

MT6: A solution of the disodium salt of N(γ - carboxymethylmercaptomercuri - β - methoxy)propyl camphoramic acid. N(γ-hydroxymercuri-β-methoxy)propyl camphoramic acid was synthesized from camphor, allyl amine and mercuric acetate by methods described in the literature. A solution of the sodium salt of this acid was then added to an aqueous solution of sodium thioglycollate. The resulting solution of the mercaptide was brought to pH 7 to 8 by addition of sodium hydroxide.

Unless otherwise stated, all of the above drugs were used at a concentration of approximately 39 mg of mercury per cc.

Procedure. Normal adult cats of both sexes weighing between 2 and 4 kg were used in this study. The animals were anesthetized with 0.5 cc per kg of dial (10%) with urethane (40%) half of which was given intramuscularly and half intraperitoneally. When necessary, anesthesia was completed by cautious intravenous injection of pentobarbital sodium solution. Electrocardiograms were recorded from lead II. A control tracing was taken and the undiluted solution of the mercurial was immediately injected into the saphenous vein. The total volume was injected over 30 seconds in the case of vol-

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* The investigation of the comparative cardiac toxicity of the mercurial diuretics was made with the assistance of grants from the Committee on Therapeutic Research of the Council on Pharmacy and Chemistry of the American Medical Association, from Lakeside Laboratories, Inc., Milwaukee, Wis., and from Campbell Products, Inc., New York City. The preparation and study of the pharmacology of N(γ-carboxymethylmercaptomercuri-β-methoxy)propyl camphoramic acid disodium salt was assisted by a grant from Campbell Products, Inc.

[1] DeGraff, A. C., and Nadler, J. E., *J. Am. Med. Assn.*, 1942, **119**, 1006.

[2] Wexler, J., and Ellis, L. B., *Am. Heart J.*, 1944, **27**, 86.

[3] Pines, I., Sanabria, A., and Arriens, R. T. H., *Brit. Heart J.*, 1944, **6**, 197.

[4] Volini, I. F., Levitt, R. O., and Martin, R. J., *J. Am. Med. Assn.*, 1945, **128**, 12.

[5] DeGraff, A. C., and Lehman, R. A., *J. Am. Med. Assn.*, 1942, **119**, 998.

[6] Long, W. K., and Farah, A., *J. Pharmacol. Exp. Therap.*, 1946, **88**, 388.

TABLE I.
Effect of Intravenous Injection of Mercurial Diuretics on the QRS Interval.

Mercuzanthin			Salyrgan-Theophylline			Mercuhydrin			Mercuric Chloride		
Dose in cc per kg	Control QRS sec.	Maximum QRS observed sec.	Dose in cc per kg	Control QRS sec.	Maximum QRS observed sec.	Dose in cc per kg	Control QRS sec.	Maximum QRS observed sec.	Dose in cc per kg	Control QRS sec.	Maximum QRS observed sec.
.035	.03	NC	.058	.04	NC	.05	.02	NC	.01*	.03	NC
.035	.03	NC	.058	.04	NC	.05	.03	NC	.04*	.03	NC
.071	.03	NC	.117	.03	.12	.1	.03	NC	.05	.03	.08
.071	.02	.04	.117	.03	.05	.1	.03	NC	.1	.03	.16
.142	.03	.20	.233	.03	.16	.2	.03	.06	.2	.02	.05
.142	.03	.14	.233	.02	.14	.2	.02	.10			
.283	.06	.34	.456	.03	.08	.4	.02	.12			
.283	.03	.10	.456	.03	.10	.4	.02	.14			
.566	.02	.12				.8	.03	.12			
.566	.03	.14				.8	.04	.10			

NC—No change.
* 0.4% solution.

umes up to 0.8 cc per kg. For the large volumes required in the case of MT6, a burette was used and the solution injected at approximately 2 cc per kg per minute. Tracings were then taken at intervals of 1, 2, 3, 5, 10, 15, 20, 30, 40, 50 and 60 minutes after injection. In some cases this series was extended up to 6 hours at 15-minute intervals.

Results. The observations have been summarized in Table I. In every case the initial dose was low enough so that no change in the electrocardiogram occurred within an hour after injection. This was then increased by a factor of 2 until a lethal or severely toxic level was reached. In the case of Salyrgan-Theophylline, Mercuhydrin, Mercuzanthin and mercuric chloride, the sequence of events following injection of a toxic dose was quite similar. The most significant changes appeared immediately after injection and could for the most part be attributed to depression of conduction. They usually consisted of atrioventricular dissociation or ventricular tachycardia and were invariably accompanied by a marked increase in the QRS interval. In some cases ventricular fibrillation and death ensued. Occasionally the ventricular rate progressively slowed until complete arrest. Usually, however, the electrocardiogram reverted in less than 30 minutes to a more or less regular sinus rhythm (Fig. 1 and 2). These phenomena are in general agreement with those reported by Volini[4] for 2 patients on whom electrocardiograms were taken during the acute episode following the injection of a mercurial diuretic. Furthermore, the literature[1–4] indicates that the typical reaction in man comes on immediately after the injection. Therefore, in the absence of direct evidence, it will be assumed that the action of these drugs in the anesthetized cat is qualitatively the same as in man. In the case of MT6 no significant changes in the tracing were observed immediately in any dose used. The highest doses gave rise to a gradual depression of conduction and rate leading to cardiac arrest after a variable period. These changes are illustrated in Fig. 3. In Table II the

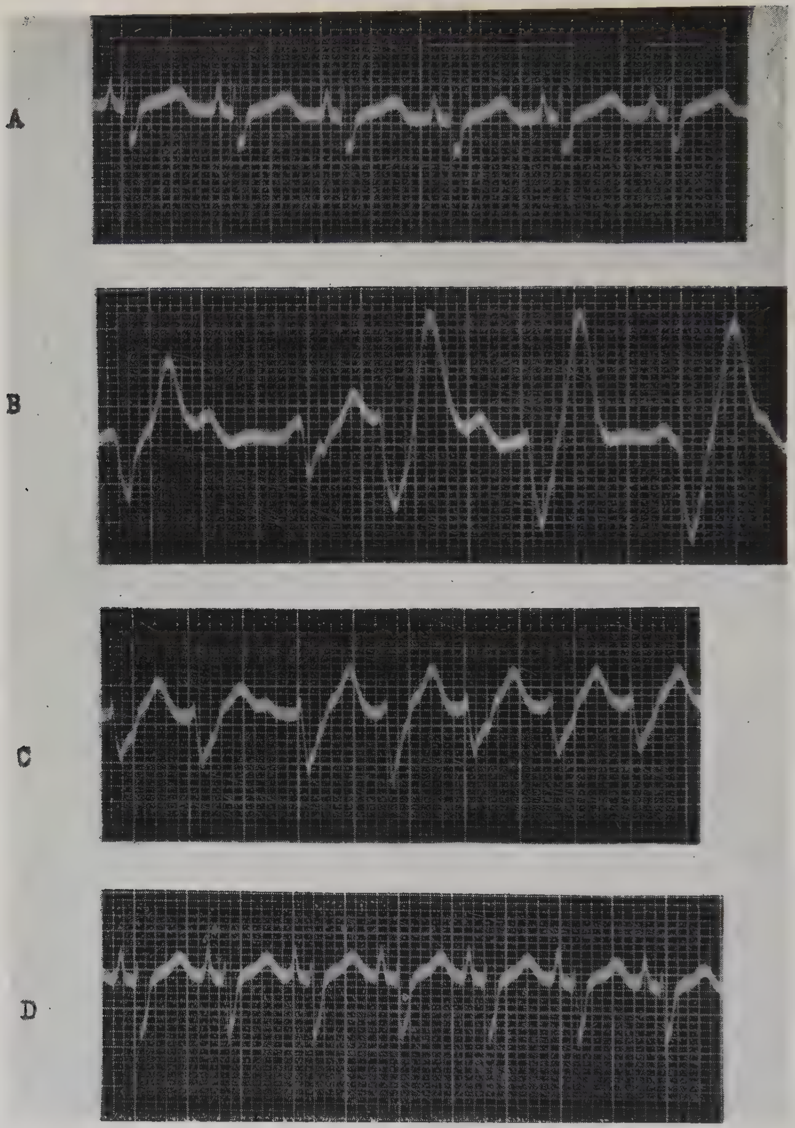

Fig. 1.
Effect of intravenous injection of 0.283 cc per kg of Mercuzanthin solution on the electrocardiogram of the anesthetized cat. A, control tracing; B, one minute after injection; C, 10 minutes after injection; D, 50 minutes after injection.

time of onset of marked electrocardiographic changes due to MT6 is given as a function of dosage. It is evident that the higher the dose the more rapidly the cardiac effects appear. However, at 4 cc per kg no cardiac toxicity appeared within 6 hours after injection. It is not to be inferred, however, that MT6 or indeed any of the mercurials used in this study can be tolerated indefinitely at these large doses. The majority of the animals died, or were expected to die, after several days due to mercury intoxication.

Quantitatively, the toxic effect of the first 4 mercurials is most consistently reflected in the electrocardiogram in an increase in the QRS interval. Using this as a criterion, the maximum tolerated dose for each drug has been roughly estimated and is given in Ta-

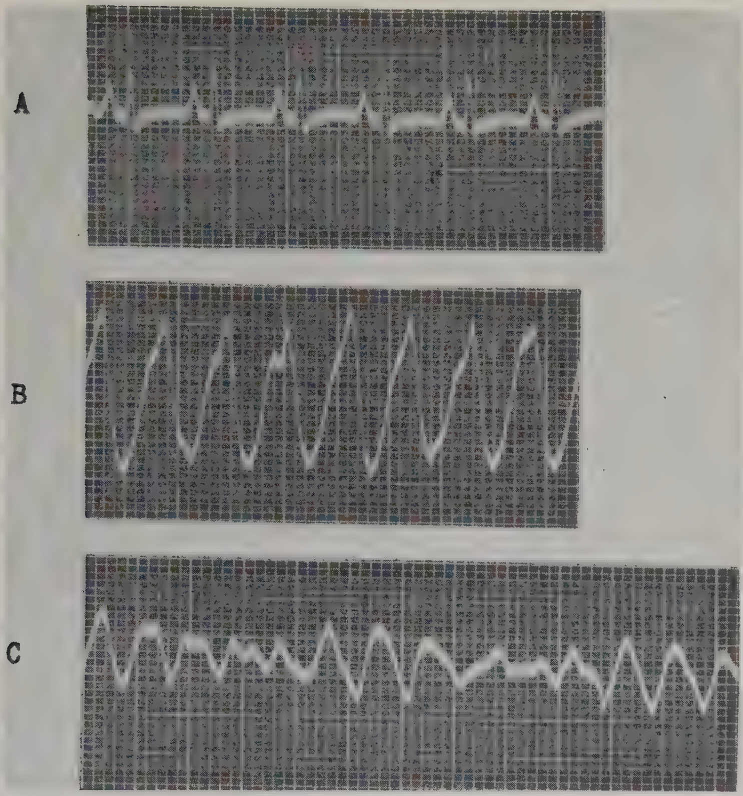

Fig. 2.
Effect of intravenous injection of 0.233 cc per kg of Salyrgan-theophylline solution on the electrocardiogram of the anesthetized cat. A, control tracing; B, one minute after injection; C, 3 minutes after injection. Death ensued shortly after C.

TABLE II.
Onset of Electrocardiographic Changes After MT6.

Dose in cc per kg	Time in minutes for onset of major E.K.G. changes (other than alterations in rate or amplitude of deflections.)	Period of observation in min
2	none	60
2	,,	60
4	,,	90
4	,,	60
4	,,	360
8	75	75
8	none	60
8	105	120
16	10	30
16	50	50
16	90	90
16	60	75

ble III. It will be noted that there is comparatively little difference in toxicity among the 3 commonly used mercurial diuretics. Furthermore, mercuric chloride, while clearly the most toxic substance studied, is closer to the diuretics than might have been anticipated. The toxicity of MT6 when judged in terms of the immediate electrocardiographic changes described above is less than 1/400 that of mercuric chloride or 1/160 that of Mercuhydrin, which is otherwise the least toxic of the group.

Discussion. Johnston[7] showed that sodium thiosulfate could restore the normal force and rhythm of the isolated turtle heart poisoned with Salyrgan or Mercuzanthin. In a previous report from this laboratory[5] it was indicated that thiosulfate exerts a similar action in the intact cat. More recently Long and Farah[6] have investigated the prophylactic and therapeutic action of 2 monothiols

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[7] Johnston, R. L., *J. Lab. Clin. Med.*, 1941, **27**, 303.

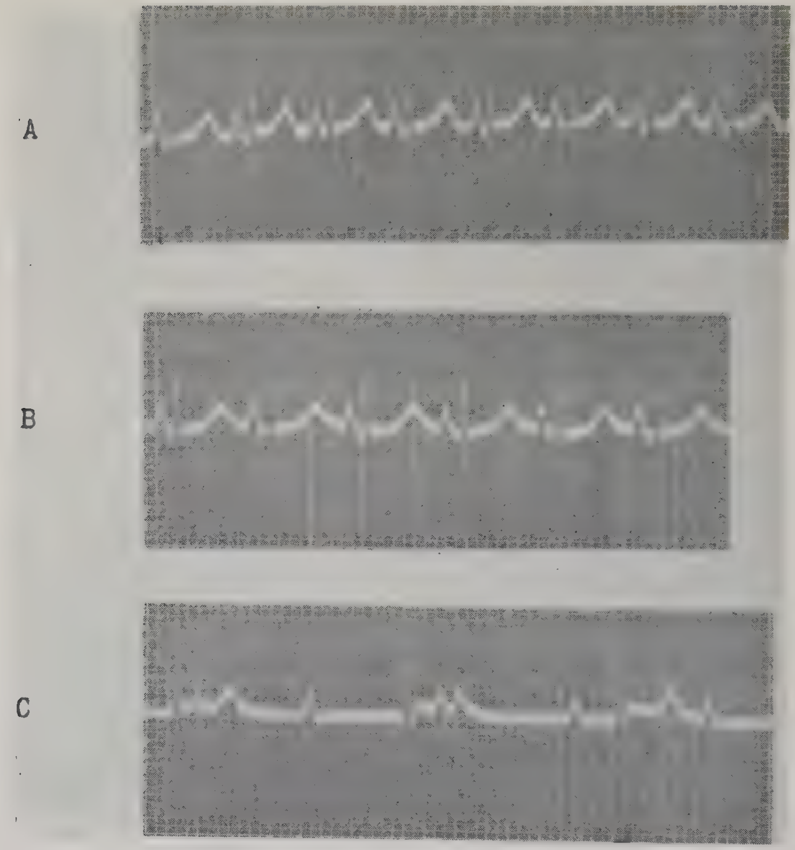


Fig. 3.
Effect of intravenous injection of 16 cc per kg of MT6 solution on the electrocardiogram of the anesthetized cat. A, control tracing; B, 40 minutes after injection; C, 50 minutes after injection. The animal died shortly after tracing C.

TABLE III.
Comparative Acute Cardiac Toxicity of Mercurials.

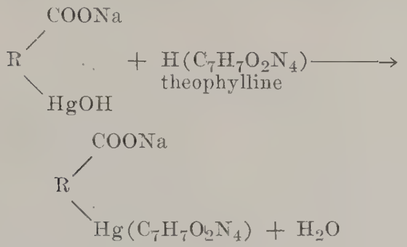
Compound	Max. tolerated intravenous dose in cc per kg based on widening of the QRS interval	Ratio
Mercuric chloride	0.04	1.0
Salyrgan-Theophylline	0.058	1.4
Mercuzanthin	0.071	1.8
Mercuhydrin	0.1	2.5
MT6	>16.0	>400.0

(cysteine and glutathione) and one dithiol (2,3-dimercapto propanol, British Antilewisite) against the acute cardiac effects of Salyrgan. They found that the intravenous LD_{50} of Salyrgan in mice was slightly increased by intravenous injection of a mol equivalent of any of the above compounds one minute before the mercurial. They also reported that arrythmias produced in the isolated heart lung preparation of the dog as well as the intact dog could be successfully treated with these compounds. 2,3-Dimercapto propanol was considerably more effective than monothiols in the intact dog but not in the isolated heart. Apparently no attempt was made to combine the mercurial with the thiol before injection and compare its toxicity with the parent drug.

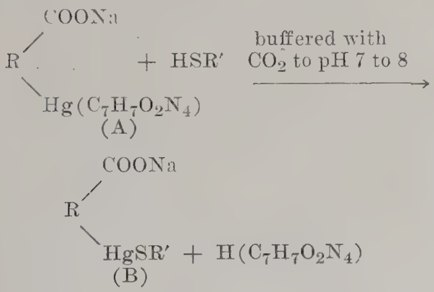
It has long been known that compounds of the general structure RHgOH will react with many simple salts as follows:

$$\mathrm{RHgOH} + \mathrm{KBr} \longrightarrow \mathrm{RHgBr} + \mathrm{KOH}$$

where the base formed may be quantitatively titrated. Presumably such a reaction goes to completion because of the failure of RHgBr to ionize. The common mercurial diuretics are prepared by a similar reaction:



It has been previously shown[8] that this combination with theophylline results in a decrease in the local toxicity of the mercurial and perhaps a slight improvement in the acute cardiac toxicity.[5] Further experiments showed that the following reaction:



will cause the precipitation of theophylline in practically theoretical yield thus suggesting that the mercaptide is more stable than the theophylline complex. The experiments described above correspondingly demonstrate a very considerable decrease in the cardiac toxicity for a compound of structure B (MT6) as compared with compounds of structure A (Mercuhydrin, Mercuzanthin, Salyrgan-Theophylline). These results are quite out of line with those of Long and Farah[6] in which treatment with the thiol preceeded the injection of the mercurial. This might have been expected, however, because in their experiments the thiol would be required to compete for the mercurial with whatever group the mercurial combines with in the tissues. The difference they observed in the effectiveness of 2,3-dimercapto propanol as compared with simple monothiols may then be caused by a difference in the fate of the detoxifying agent.

A number of reports have recently appeared concerning the general subject of detoxification of heavy metals by formation of mercaptides, especially in connection with the development of British Antilewisite. Most of this work is not relevant to the acute action of mercury on the heart and need not be considered here. Moreover, it should be noted that the magnitude of detoxification demonstrated in the above experiments is not necessarily capable of wide application. Thus the acute intravenous lethal doses of a series of alkyl mercuric thioglycollates (also defined by structure B) reported by Cohen[9] are only slightly greater than the maximum tolerated doses given in Table III for the 3 diuretics.

Clinical studies with MT6 will be reported elsewhere.

Summary. Mercuric chloride, Salyrgan-theophylline, Mercuzanthin, Mercuhydrin and N(γ-carboxymethylmercaptomercuri-β-methoxy)propyl camphoramic acid disodium salt (MT6) have been compared with respect to their ability to cause cardiac toxicity as manifested by changes in the electrocardiogram of the cat. The toxicity has been found to decrease in the order named. Compound MT6 causes no immediate changes in the electrocardiogram in doses up to 160 times the maximum tolerated dose of Mercuhydrin. This improvement appears to be due to the relatively greater stability of the mercaptide as compared with the theophylline complex.

The author wishes to express his thanks to Doctor A. C. DeGraff and Doctor R. C. Batterman for their advice and cooperation and to the Misses Alice Sullivan and Jean Lippold for technical assistance.

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[8] DeGraff, A. C., Batterman, R. C., and Lehman, R. A., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 373.

[9] Cohen, S. J., *J. Pharm. Exp. Therap.*, 1929, **35**, 343.

15819

Effect of the Purified Blood Group A Substance on Permeability of the Capillaries.

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(Introduced by Albert E. Sobel.)
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In a previous communication,[1] it was shown with the aid of a new method that a number of substances increase the permeability of the capillaries to a very high degree. Very active were commercial peptones. Some of the constituents of commercial peptones were examined, therefore, and in the course of these investigations it was found that A and B substances of Witebsky had a strong effect on capillary permeability. It could not be decided, however, whether this effect was due to the blood substances themselves or to impurities. To answer this question we first tried to make use of the observation that enzymes in saliva and feces destroy the blood group substances.[2-5] We, therefore, treated the AB substance of Witebsky with extracts of feces and the saliva of non-secretors and examined whether the effect on capillary permeability disappeared with the blood group activity. These attempts, however, were frustrated by the fact that the extracts of feces and the saliva of non-secretors themselves had a very strong effect on capillary permeability. A new avenue of approach was opened by the work of Kabat, Bendich and Bezer[6,7] who succeeded in purifying the blood group A substance. The capillary effect of this substance will be described in the present paper. Included are investigations on an inactive substance, likewise isolated by Kabat, which is chemically and physico-chemically indistinguishable from the A substance but differs from it serologically. The nature of this substance will be discussed below.

Methods and Materials. The method used in this investigation for the demonstration of the capillary effect is the indirect intracutaneous test with diphtheric toxin and the indirect intramuscular test with tetanic toxin. Both procedures have in common that the increased capillary permeability manifests itself in the increased effect of the antitoxin. The details of this method, and the evidence for this interpretation of the experimental results were presented in our previous communication.[1]

The preparation of the purified A substance has been described by Kabat, Bendich, and Bezer.[6,7] Gastric mucin or stomach linings of hogs were digested with pepsin and purified with the aid of the method of Morgan

TABLE I.
Capillary Effect of the Purified Blood Group A Substance as Demonstrated by the Indirect Skin Test with Diphtheric Toxin.

Dilution of toxin	Series A (control)	Series B
1:50	N	—
1:100	N	E++
1:200	E++++	E+
1:400	E+++	E+
1:800	E++	O
1:1,600	E++	O
1:3,200	E+	O
1:6,400	O	—
1:12,800	O	—

N: necrosis.
E: erythema.
Number of + indicates extent of erythema.
O: no reaction.
—: not done
Results read after 72 hr.

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[1] Friedemann, U., Traub, F. B., and Langstadt, D., *J. Immunol.*, 1946, **54**, 197.

[2] Schiff, F., and Akune, M., *Münch Med. Wochensch.*, 1931, 657.

[3] Stimpfl, A., *Z. f. Immunitätsf.*, 1932, **76**, 159.

[4] Schiff, F., and Weiler, G., *Biochem. z.*, 1931, **235**, 454.

[5] Schiff, F., and Weiler, G., *Biochem. z.*, 1931, **239**, 489.

[6] Kabat, E. A., Bendich, A., and Bezer, A. E., *J. Exp. Med.*, 1946, **83**, 477.

[7] Bendich, A., Kabat, E. A., and Bezer, A. E., *J. Exp. Med.*, 1946, **83**, 485.

TABLE II.
Capillary Effect of the Purified Blood Group A Substance and of a Preparation Containing 60% A Substance and 40% "Inactive Substance" as Demonstrated by the Indirect Test with Tetanic Toxin.

Dilution of antitoxin	Series A (control)	Series B	Series C
1:20	+6, L.T. 1	—	—
1:40	+6, L.T. 1	—	L.T. 2, O
1:80	+3, +3	—	L.T. 2, O
1:160	+2, +6	L.T. 1 L.T. 2	L.T. 2 L.T. 5
1:320	—	L.T. 1 L.T. 4	+5, +8
1:640	—	+2, G.T. 4	+3, +6
1:1,280	—	+5, G.T. 4	+3, +3

—: not done.
O: no symptoms.
L.T.: local tetanus.
G.T.: general tetanus.
+: indicates death
Numerals indicate day of onset of tetanus or of death.

and King.[8] The stomachs of some hogs yielded the pure A substance while from other stomachs a second substance was obtained, chemically identical with the A substance but serologically inactive. Finally, from the pooled hog stomachs was obtained a product composed of 60% of A substance and 40% of inactive substance. These 3 preparations were kindly placed at our disposal by Dr. Kabat. In the following experiments 1% solutions of these substances in saline were used.

Experiment 1. One cc of diphtheric antitoxin 874 (1600 units per mil.) in a dilution 1:50 was injected intravenously into a white rabbit weighing 2500 g. Immediately afterward 0.1 cc of serial dilutions of diphtheric toxin 1116 were injected intracutaneously. The serial dilutions of toxin were injected in duplicates. In Series A the toxin was diluted in saline solution, in Series B it was diluted in a 1% solution of the purified A substance. The results are recorded in Table I.

It will be seen from Table I that at least 8 times more toxin has been neutralized in Series B than in Series A. This result we interpret as indicating that the purified A substance increases the permeability of the capillaries of the skin to antitoxin approximately 8 times.

Experiment 2. 0.5 cc of serial dilutions of tetanic antitoxin 327 (1200 units per mil.) was injected intravenously into guinea pigs weighing 250 g. Twenty lethal doses of tetanic toxin 1556 (0.1 mil. of a dilution 1:25) were injected intramuscularly. The experiments were carried out 3 times. In Series A the toxin was diluted in saline solution, in Series B in a 1% solution of the purified A substance and in series C in a solution of the preparation containing 60% A substance and 40% inactive substance. The results are recorded in Table II.

Again in Series B the antitoxin is approximately 8 times more effective than in Series A. In Series B the minimal concentration which protected both animals against death was 1:320 while in Series A even a concentration 1:40 protected only one animal. In Series C the antitoxin was approximately 4 times more effective than in Series A. These results indicate that a 1% solution of the purified A substance increased the permeability of the capillaries to antitoxin about 8 times while with a 1% solution of the preparation containing 60% A substance and 40% "inactive" substance the increase was about 4 times.

Experiment 3. One cc of diphtheric antitoxin 874 (1600 units per mil.) was injected intravenously into a white rabbit weighing 2500 g. 0.1 cc of serial dilutions of toxin 1116 was injected intracutaneously. In Series A the toxin was diluted in saline, in

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[8] Morgan, W. T. J., and King, H. R., *Biochem. J.*, 1943, **37**, 640.

TABLE III.
Capillary Effect of the "Inactive Substance" as Demonstrated by the Indirect Skin Test with Diphtheric Toxin.

Dilutions of toxin	Series A (control)	Series B
1:50	N	—
1:100	N	N
1:200	E++++	E++++
1:400	E+++	E+++
1:800	E++	E+
1:1,600	E+	O
1:3,200	E+	O
1:6,400	O	—
1:12,800	O	—

N: necrosis.
E: erythema.
No. of + indicates extent of erythema.
O: no reaction.
—: not done.
Result read after 72 hr.

Series B in a 1% solution of the "inactive" substance. The results are recorded in Table III.

As may be seen from Table III, the inactive substance increases the permeability of the capillaries 4 times.

Discussion. The scarcity of the material at our disposal made experiments on a larger scale impossible. The quantitative data, therefore, require confirmation when larger quantities of the purified blood group substances will be available. The indirect intracutaneous test with diphtheric toxin as well as the indirect test with tetanic toxin, however, leaves no doubt that the purified A substance increases the rate of neutralization of toxins, presumably resulting from the increase of the permeability of the capillaries. The fact that the "inactive" substance likewise increased the rate of neutralization and thus presumably had affected the capillary permeability, although to a lesser degree, seems at first to detract from the significance of our findings. Dr. Kabat, however, has authorized us to publish the following personal communication:

"Kabat, Bendich and Bezer[9] have found that Type XIV antipneumococcal horse serum, which has been shown to agglutinate cells of groups A, B and O,[10] gives precipitin reactions with both the active and the inactive hog substances, leading them to the inference that the inactive substance may be associated with blood group O activity." It is highly probable, therefore, that the capillary effect of the "inactive" substance is due to its relationship to the blood group O substance. The blood group B substance is not available in a purified form and nothing can be said, thus far, as to its effect.

As shown in our previous communication,[1] the capacity of increasing capillary permeability is common to a wide variety of substances. To these we now have added the purified blood group substances. This finding assumes additional significance in view of the presence of these substances in the tissue fluids of secretors. Schiff,[11] and Friedenreich and Hartman[12] have discussed the question whether these substances may fulfill any physiological function. On the basis of the results reported in the present paper, the possibility must be considered that they may play a role in the regulation of capillary permeability. From this point of view the division of human beings into secretors and nonsecretors gains added interest.

Summary. With the aid of a new method it was found that the purified blood group A substance considerably increases capillary permeability. Similar results were obtained with the "inactive" substance purified by Kabat *et al.* from hog stomach, and probably closely related to the group O substance.

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[9] Personal communication.

[10] Weil, A. J., and Sherman, E., *J. Immunol.*, 1939, **36**, 139.

[11] Schiff, F., *Über die blutgruppenspecifischen substancen des menschlichen Koerpers*, Gustav Fischer, Jena, 1931.

[12] Friedenreich, V., and Hartmann, G., *Z. f. Immunitatsf.*, 1938, **92**, 141.

15820

Reaction of the Human Body Louse: (*Pediculus humanus corporis*) to the Ingestion of Guinea Pig Blood.

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There seems to be no doubt that man apparently is the only natural host of the body louse. Human body lice have occasionally been found on domestic animals but, as Nuttal noted[1] ". . . this fact has the same importance as finding a louse on the hearth-rug."

Charles Nicolle[2] on the other hand, in experimental typhus fever studies, had no difficulty in feeding lice repeatedly on monkeys *(Macacus rhesus)* and found that a certain proportion of lice when fed on monkeys will remain alive for as long as 20 days. However, Bacot and Segal[3] found only rare survivors among lice fed on monkeys for 4 successive days.

While some successful attempts have been reported of feeding body lice on rabbits, guinea pigs, mice, fowl, pigeons, etc., many more failures are recorded. It has often been claimed that blood of certain animals (particularly of rodents) is toxic to the genus *Pediculus* but, as Buxton noted,[4] no one yet has conducted experiments comparing the longevity of lice given a single blood meal on guinea pigs or rats, or on man, to that of unfed lice.

Thus, the possibility exists that the blood of animals, other than of man, is not toxic to the body louse, but that the body louse is able to digest human blood because its digestive enzymes are specific only for human red blood cells.

The following experiments, conducted at the Pasteur Institute of Tunis, seem to confirm this hypothesis. Only one species of rodent, the guinea pig was used.

Experiment I: One hundred female lice from the stock laboratory colony were divided into 2 lots of 50 lice each (A and B). The lice were placed in an incubator at 30-32°C, and kept unfed for 30 hours in order to clear their intestines of the last human blood-meal. Lice of Lot A then were fed on the arm of a human volunteer while lice of Lot B were fed on the shaven and cleansed flank of a guinea pig. After feeding for one hour, the lice of both lots were seen to be fully engorged with blood, as indicated by the large red spot in their mid-gut visible through the integument. The lice of both lots were then returned to the incubator (30-32°C). At hourly intervals thereafter a few of the lice were withdrawn from the incubator, dissected in a drop of saline and examined microscopically with the following results:

Lot A. After one hour the lice showed a great number of intact red blood cells although it was observed that some red cells already had been destroyed as evidenced by the presence of a chocolate-brown amorphous mass. After 2 hours the number of intact red blood cells was decreased still further and by the end of 4 hours all the red cells had been destroyed. The spot in the mid-gut at this period appeared dark chocolate-brown, or almost black in color.

Lot B. The red blood cells remained intact in appearance until the death of the lice which generally took place about 48 hours after the ingestion of guinea pig blood. The intestines were still full of unchanged blood. A large number of lice died showing signs of ruptured intestines or radically altered permeability of the intestines. The red color, limited at first to the intestines, diffused progressively into the abdominal cavity, into the legs and even into the extremities of the antennae until the entire louse was red in color with a darker spot present in the region of the mid-gut. This phenomenon occurs to a certain extent in normal louse colonies[4] but appears rather more frequently among lice fed on guinea pig blood.

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* Associate member of Pasteur Institute of Tunis.

[1] Nuttall, G. H. F., *Parasitology*, 1917, **10**, 80.

[2] Nicolle, C., *Ann. Inst. Pasteur*, 1910, **24**, 243.

[3] Bacot, A. W., and Segal, J., *Brit. J. Path.*, 1922, **3**, 125.

[4] Buxton, P. A., *The Louse*, 1939, 2nd edition, Edward Arnolds, London.

The above observations indicate that it is apparently impossible for the body louse to digest guinea pig red blood cells. It is believed that the intestines are ruptured by their exertions to destroy or eliminate the guinea pig red blood cells.

Experiment II. Three lots of 30 lice each (15 males and 15 females) 12-15 days of age, were isolated in separate boxes marked A, B and C. The boxes were placed in an incubator at 30-32°C, and the lice were held unfed for an average period of 36 hours. At the end of that time the lice were devoid of blood as could be seen through the integument. The following experiments were then performed:

Box A. The lice were fed on man for one hour. They were then returned to the incubator and held without further feeding until death occurred, as follows:

24 hrs.—29 lice alive; all fully engorged (1 male died prior to feeding)
48 " —29 lice alive; digestion of the blood meal completed
72 " —2 males and 2 females dead; 25 lice alive, intestines completely empty
96 " —no changes observed
120 " —all lice dead

Box B. These lice were fed on the shaven, cleansed flank of a guinea pig for one hour, and then stored unfed in the incubator (30-32°C) until death occurred as follows:

24 hrs.—30 lice alive; all fully engorged; no visible digestion of blood apparent
48 " —2 males and 4 females dead, the remaining lice move with difficulty; a large number show signs of ruptured intestines
72 " —all lice dead; the intestines are still filled with blood of normal appearance

Box C. These lice which had already been starved for an average period of 36 hours, were held unfed in the incubator (30-32°C) until death occurred, as follows:

24 hrs.—30 lice alive; intestines completely empty
48 " —1 female and 4 males dead
72 " —all lice dead

An analysis of the above data indicates that: (1) Lice starved for an average of 36 hours, then fed only once on man, remain alive for approximately 4 additional days. This is the normal survival time of unfed lice under optimal conditions. (2) Lice starved for 36 hours following a human blood meal, and then allowed to feed once on a guinea pig, all die by the third day after ingestion of the guinea pig blood. Hence the time elapsed from the last human blood meal to the death of the experimental lice is approximately 4 to 4½ days. Thus, the total survival time of the lice (dating from the time of the last human blood meal) which fed on guinea pig blood (Box B) is approximately the same as that of lice which were starved for the same length of time but then fed a single time on human blood (Box A), or not fed at all (Box C).

Experiment III. Fifty 13-day-old lice were isolated in a box and held unfed in an incubator (30-32°C) for 33 hours. One died during this period. The remaining 49 lice were fed on a guinea pig for one hour and when fully engorged were placed in an incubator at 37°C for 2 hours. The purpose of this was to induce rapid dehydration and reduction of the guinea pig blood meal, permitting immediate feeding of the lice upon their withdrawal from the incubator. They were then fed on man for one-half hour. Subsequent half-hour human blood feedings were continued twice daily, morning and evening. All lice in this experiment remained alive until natural death occurred on the 35th to 40th day.

The above observations seem to confirm the hypothesis that guinea pig blood *per se* is not toxic to the body louse, since lice that have fed once on a guinea pig live their normal life span when they are allowed to continue their daily feedings on human blood.

Summary and Conclusions. Contrary to general belief, the experiments reported above indicate that guinea pig blood *per se* is not toxic to the human body louse.

In the first experiment it was demonstrated that the red blood cells of guinea pigs remain undigested in the gut of the louse.

In the second experiment it was shown that lice starved for an average period of 36 hours and then fed once on a guinea pig, die within 72 hours, apparently of starvation. Control lice fed only on human blood, likewise were found to die 4 to 4½ days following their last human blood meal.

In the third experiment it was shown that lice fed once on guinea pig blood live their normal life span when their daily feedings are continued subsequently on human blood.

It would appear that human body lice secrete a digestive enzyme specific only for human red blood cells which makes it impossible for them to digest guinea pig blood.

15821

Mass Infection of Body Lice with *Rickettsia prowazeki.*

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In a preceding article[1] it was reported that body lice *(Pediculus humanus corporis)* which have engorged on guinea pig blood are unaffected and remain alive for their normal life span if subsequent feedings are continued almost immediately (within 2 hours) on human blood. As a result of this observation, infection of human body lice with *Rickettsia prowazeki* was achieved on a large scale, following a single meal on an infected guinea pig.

The inconveniences and disadvantages of infecting lice by the rectal route, the method employed by Weigl[2] for the preparation of typhus vaccine, are well known. Infection of body lice by feeding them on human typhus cases, while feasible, is nonetheless rather impractical. Guinea pigs, on the other hand, may be easily infected and maintained in the laboratory. The advantages of feeding lice on infected guinea pigs, followed by feeding the lice subsequently on *typhus immune humans,* for the ultimate preparation of typhus vaccine are obvious.

Method of infection. Fifty female lice 12-13 days of age were starved for 30 hours. These lice, as could be seen through the integument, were devoid of blood. They were then allowed to feed on a guinea pig infected 10 days previously with the Tunis strain of classical louse-borne typhus.

The flank of the guinea pig had been shaved and rubbed with alcohol, as lice often refuse to feed if the guinea pig is not prepared in this manner. In 30 minutes the lice were fully engorged, as indicated by the large red spot visible in the mid-gut.

The lice were then placed for 2 hours in an incubator at 37°C, in order to induce rapid dehydration and reduction in volume of the engorged guinea pig blood. They were then allowed to feed on a typhus-immune man for one-half hour. Subsequent human blood feedings on a typhus-immune individual were continued twice daily. By the end of the 8th day, 37 of the 50 lice were still alive, while 13 had succumbed.

A number of the surviving lice were dissected and preparations of their intestines were stained with a mixture of May-Grünwald-Giemsa. Microscopic examination of these preparations showed, without exception, rickettsiae in large numbers. The following experiments were carried out to identify the rickettsiae present.

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* Associate member of Pasteur Institute of Tunis.

[1] Cabasso, V., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 437.

[2] Weigl, R., *Arch. Inst. Pasteur Tunis,* 1933, **22**, 315.

Experiment I. Excreta of the infected lice, collected between the 8th and 10th days following the infective meal, were suspended in saline and inoculated intraperitoneally into 4 guinea pigs (51B, 58N, 62N and 64N). Three guinea pigs showed signs of classical typhus 8 to 13 days following the inoculation; the fourth (64N) died prematurely.

Forty days after the infective inoculation, guinea pigs 51B and 58N were immune to a challenge inoculation with a guinea pig brain suspension of the Tunis strain of typhus virus. Guinea pig 62N was sacrificed on the 10th day; the brain was triturated, suspended in saline, and injected intraperitoneally into guinea pigs 72N and 73N. Both of these latter guinea pigs showed typical clinical signs of typhus beginning on the 6th day and were immune to the challenge inoculation given 40 days later.

Experiment II. The intestines of 10 infected lice were triturated, suspended in saline and inoculated intraperitoneally into guinea pigs 68N and 69N. Both animals showed signs of classical typhus on the 6th day.

Guinea pig 68N was sacrificed on the 10th day and a suspension of its brain inoculated intraperitoneally into guinea pigs 74N and 75N. Both showed signs of classical typhus on the 6th and 7th days respectively, and were immune to the challenge inoculation on the 40th day.

Experiment III. Ten intact lice were sterilized externally with 70% alcohol, ground in a mortar and suspended in saline. The suspension was inoculated intraperitoneally into guinea pigs 50B, 52B, 65N and 66N. On the 2nd day following inoculation all 4 guinea pigs showed severe signs of typhus and all showed signs of pronounced cachexia at the end of the 5th day.

Guinea pig 50B was sacrificed on the 10th day and a suspension of its brain inoculated intraperitoneally into guinea pigs 55N and 56N. Guinea pig 55N died prematurely while 56N showed signs of typhus on the 7th day and was sacrificed on the 12th day. A suspension of its brain was inoculated intraperitoneally into guinea pigs 60N and 61N, both of which showed signs of typhus on the 6th day and were immune to the challenge inoculation on the 40th day.

Guinea pigs 65N, 66N and 52B died on the 7th, 7th and 16th days, respectively, following inoculation. On autopsy the organs showed no abnormal appearance except that the viscera were covered with a glossy white, membranous-like film. Smears of this material when stained with Giemsa showed innumerable rickettsiae, both free or enclosed in protoplasm of endothelial cells.

The virus which proved to be extremely virulent on direct inoculation of infected lice into guinea pigs, assumed its normal characteristics with the usual incubation period on the first passage from guinea pig to guinea pig.

Summary and Conclusions. The experiments reported above indicate that human body lice fed once on a typhus-infected guinea pig at the height of the febrile period, and subsequently fed on a typhus-immune human, develop massive infection with *Rickettsia prowazeki.* In comparison to the rectal inoculation of lice for the preparation of the Weigl type typhus vaccine, this method of infecting lice seems to offer certain advantages.

15822

Response of Anemic Chicks to Peteroylglutamic Acid.*

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In the course of studying the quantitative requirements of chicks for pteroylglutamic acid (folic acid),[1] information was desired regarding the ability of anemic chicks to make effective use of this substance in order to compare their response to that of anemic monkeys reported by Day and associates,[2] and that of human subjects described by Spies and co-workers.[3]

Experimental and Results. Exp. 1. Surviving chicks of several experiments were divided at random into 2 groups of 12 chicks each. These chicks had been reared to 4 weeks of age on Diet 653 described by Hill and associates.[4] Microbiological assays indicated that the diet contained about 2 μg of free pteroylglutamic acid and about 3 μg in the conjugate form per 100 g of diet. The hemoglobin levels of these chicks averaged 2.7 g per 100 cc of blood with a range of 0.6 to 6.2 g per 100 cc.

The chicks in one group were injected intramuscularly with 100 μg of pteroylglutamic acid;† those in the other group were maintained as controls. Hemoglobin values and percentage of reticulocytes were determined at 2, 4, 6, 9, 11, 13 and 16 days following the single injection of pteroylglutamic acid.

In the group receiving the vitamin, 3 chicks died the day injections were made but no other mortality occurred during the experimental period. In the control group, 6 chicks died during the first 2 days and only 2 were alive at the end of the experiment.

The percentage of reticulocytes was strikingly greater in the group receiving the pteroylglutamic acid. Reticulocytosis reached a peak on the 6th day following injection after which it subsided rapidly to less than 10%, which is the level usually observed in untreated chicks. The peak of reticulocytosis in all cases was reached before the 9th day following the injection. These relationships are shown in Fig. 1.

Reticulocytes increased slightly in the surviving chicks in the control group. This increase may have been due to mobilization of pteroylglutamic acid in the catabolism of body tissues, or it may have been the result of normal erythropoietic stimuli occurring in this severe stage of anemia, analogous to spontaneous remission in pernicious anemia in humans.

The hemoglobin response was no less striking than the reticulocyte increase. Two days following the injection of pteroylglutamic acid, hemoglobin values had increased in most chicks. The increase was definite in all chicks by the 6th day. The peak in hemoglobin level was reached in 9 days, coincidental with the rapid decrease in reticulocytes.

Exp. 2. The purpose of this experiment was to compare oral administration with intramuscular injection of pteroylglutamic acid, and to establish the amount of the vitamin required for optimum reticulocytosis and hemoglobin formation. Seventy chicks which had been reared to 4 weeks of age on

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[1] Robertson, E. I., Daniel, Louise J., Farmer, Florence A., Norris, L. C., and Heuser, G. F., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 97.

[2] Day, P. L., Mims, V., and Totter, J. H., *J. Biol. Chem.*, 1945, **161**, 45.

[3] Spies, T. D., Vilter, C. F., Koch, M. B., and Caldwell, M. H., *So. Med. J.*, 1945, **38**, 707.

[4] Hill, F. W., Norris, L. C., and Heuser, G. F., *J. Nutrition*, 1944, **28**, 175.

† The authors are indebted to Lederle Laboratories, Inc., Pearl River, N. Y., for pteroylglutamic acid.

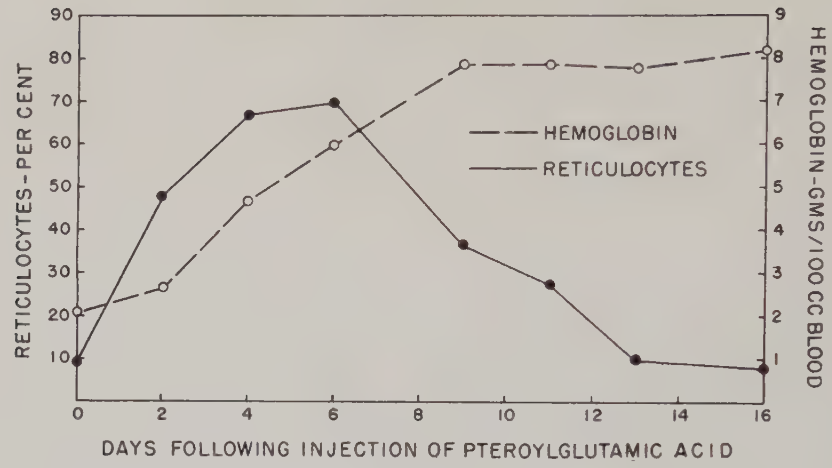

Fig. 1.
Reticulocyte and hemoglobin response of chicks to a single injection of pteroylglutamic acid.

TABLE I.
Response of Chicks to Pteroylglutamic Acid.

	Days following treatment									
Groups	Body wt (g)				Hb level (g/100 ml)				Retics. (%)	
	0	4	7	11	0	4	7	11	4	7
Basal	114	112	108	—(0)†	1.7	0.7	1.2	—	11	20
In feed*	109	129	168	220(4)	3.1	3.2	6.4	7.3	60	47
Inj. with:										
5 μg	115	143	140	163(3)	2.1	2.2	3.2	3.7	40	25
10	113	144	128	155(4)	2.4	2.8	4.1	3.4	42	26
20	109	131	148	159(5)	2.6	2.7	4.1	3.0	59	35
30	117	156	179	198(5)	2.2	3.2	5.0	6.1	63	41
40	117	136	131	158(3)	2.1	3.4	6.2	7.3	72	17
50	130	165	174	192(5)	2.3	4.4	7.5	8.0	73	39
Oral:										
5	109	131	129	132(4)	2.2	1.6	1.4	1.3	42	11
10	125	130	145	157(3)	2.8	1.4	1.7	1.8	20	25
20	118	131	131	145(3)	2.2	1.9	2.7	2.8	41	39
30	122	147	153	162(3)	1.9	2.8	3.4	3.0	56	39
40	117	144	154	156(5)	2.7	3.7	3.8	3.5	51	26
50	114	138	146	161(4)	1.9	3.5	4.6	4.0	75	39

* 500 μg of folic acid per 100 g of feed from 0 to 7 days; 250 μg thereafter.
† Numbers in parentheses indicate surviving chicks.

Diet 653 but without pteroylglutamic acid, and whose hemoglobin levels ranged between 1 and 4 g per 100 cc of blood, with weights between 100 and 150 g, were divided into 14 groups of 5 chicks each. Six groups were used for oral administration, and 6 groups for injection of pteroylglutamic acid. Of the remaining groups one was maintained as control and the other received the vitamin in its diet.

The results of Exp. 2 are presented in Table I. Reticulocytosis was greater on the 4th than on the 7th day following treatment. This applies to the chicks which received pteroylglutamic acid in their diet, and to those to which the vitamin was administered orally or by intramuscular injection.

Hemoglobin levels were greatest in the groups which received pteroylglutamic acid by injection. When the vitamin was supplied in the feed it produced a greater response in the hemoglobin level than when admin-

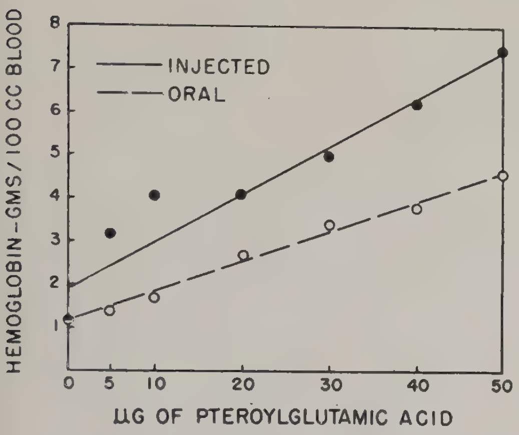

Fig. 2.
The hemoglobin response of chicks to varying levels of pteroylglutamic acid.

istered orally but separate from the feed. In all cases the response was proportional to the amount of pteroylglutamic acid administered. In the oral and injected groups the hemoglobin response was more pronounced at 7 than at 4 days following treatment. The values for the 11th day were variable due to the loss of some chicks. Hemoglobin increases after 7 days were plotted and the results are shown in Fig. 2.

Intramuscular injection of pteroylglutamic acid was approximately twice as effective in increasing hemoglobin as the same amount of the vitamin given orally. This may be due to actual destruction during the digestive processes, or to limited absorption of the vitamin.

The chicks receiving a diet containing 250 to 500 μg pteroylglutamic acid per 100 g of diet did not show increases in hemoglobin as great as that produced by the injection of 50 μg of the vitamin. Based upon feed consumption during the 7-day period the average intake per chick was estimated to be 486 μg of pteroylglutamic acid. The response of these chicks in hemoglobin increase was similar to that produced by the injection of 40 μg of the vitamin.

Discussion. The data reported for anemic chicks indicate that their response to pteroylglutamic acid is similar to that of other species. Spies and associates[3] reported peaks in reticulocyte response in 6 to 8 days following intravenous injection of folic acid in humans afflicted with macrocytic anemia in relapse. The peak of reticulocytosis rarely reached 20%. Day and co-workers[2] observed a 7 to 47% increase in reticulocytes between 4 and 7 days following the administration of *Lactobacillus casei* factor to vitamin M-deficient monkeys. The data reported here for anemic chicks show that reticulocyte response reached a peak 4 to 6 days following administration, and often exceeded 90% for individuals. It appears, therefore, that pteroylglutamic acid stimulates reticulocytosis in the chick the same as has been reported for man and monkey.

These data indicate that intramuscular injection of pteroylglutamic acid was more effective than oral administration in increasing the hemoglobin levels of anemic chicks. However, Goldsmith[5] found oral administration equally or more effective in increasing hemoglobin levels of humans with macrocytic anemia. A possible explanation of this difference may be that suboptimum amounts of the vitamin were given to the anemic chicks.

Summary. Chicks deprived of pteroylglutamic acid (folic acid) for the first 4 weeks of life respond dramatically to a single administration of the vitamin given either orally or intramuscularly. The reticulocyte response preceded hemoglobin increase and reached a peak 4 to 6 days following administration. Intramuscular injection of pteroylglutamic acid was more than twice as effective as the same amount administered orally. The hemoglobin increase in 7 days was proportional to the amount of pteroylglutamic acid administered either by mouth or by intramuscular injection.

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[5] Goldsmith, Grace A., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 115.

15823

Stability of Thrombin in the Presence of Fibrinolysin.*

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Under certain conditions blood shows marked proteolytic activity, and digests such substrates as casein, gelatine and fibrin.[1] It has recently been shown that the proteolytic activity of serum is due principally to an endopeptidase which is homospecific with an enzymatic component of papain.[2] Earlier work has demonstrated the existence of an enzyme precursor ("profibrinolysin") in blood, which is spontaneously activated following the destruction of an inhibitor by treatment with chloroform or certain other denaturing agents.[3,4] It is also activated specifically by a substance found in streptococcal cultures which is known as streptokinase.[5] To avoid confusion of terminology, the nomenclature suggested by Loomis, George and Ryder[6] has been used throughout this paper. The effect of trypsin on the clotting activity of thrombin was studied by Glazko and Ferguson[7,8] prior to the recognition of fibrinolysin as an important blood protease. The work presented here demonstrates clearly that fibrinolysin has no appreciable effect on thrombin, confirming statements made by others.[6,9] In addition it was observed that the thrombin preparation used in these experiments had some stabilizing effect on fibrinolysin. These results are of interest because of the close association of thrombin with fibrinolysin in globulin fraction III-3,[10] and of prothrombin with profibrinolysin in globulin fraction III-2.[11]

Materials and Methods. In studying thrombin stability, serial dilutions of thrombin were made in 5 ml volumes of buffered saline, containing M/100 phosphate at pH 7.4 and a small amount of phenyl mercuric borate to prevent bacterial growth. The thrombin preparation was obtained commercially as Lederle's "Hemostatic Clotting Globulin." After warming to 38°C in a water bath, 1 ml portions of enzyme solution were added to each thrombin dilution, and the mixtures were maintained at 38°C. Clotting tests were performed at regular intervals by removing 0.5 ml of each mixture and transferring to 1.0 ml portions of 0.15% fibrinogen solution at 25°C. Clotting times were measured from the moment of addition of thrombin to the first appearance of a clot.

The stability of fibrinolysin in the presence of thrombin was estimated by measurement of the time required to lyse a standard fibrin clot. Equal volumes of thrombin and fibrinolysin solutions were warmed to 38°C, mixed and tested at intervals for fibrinolytic activity by transferring 0.5 ml amounts to 0.2 ml of fresh 1:10 thrombin, and then adding 1.0 ml of saline and 0.5 ml of 0.6% fibrinogen solution. Lytic times were measured from the time of addition of fibrinogen to the time at which the gel structure of the

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* Aided by a grant from the Life Insurance Medical Research Fund.

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TABLE I.
Stability of Thrombin in the Presence of Globulin Fraction III-3.

Thrombin dilution	Incubation time (hr)							
	No protease added				With protease added			
	0	1	5	20	0	1	5	20
1:2	4	4	4	4	5	5	5	5
1:4	5	5	5	5	6	7	7	7
1:8	7	7	7	7	9	8	9	8
1:16	9	10	11	13	12	13	13	14
1:32	13	14	16	19	17	16	17	20
1:64	21	23	25	33	20	19	23	28
1:128	34	37	45	67	24	25	28	43
1:256	75	70	85	170	25	25	30	65
No thrombin added	—	—	—	—	30	32	48	40

Body of table gives clotting times in seconds.
Incubation mixture: 5 ml thrombin + 1 ml (= 10 mg) globulin III-3.
Clotting test: 0.5 ml incubation mixture + 1.0 ml 0.15% fibrinogen.

TABLE II.
Stability of Thrombin in the Presence of Fibrinolysin.

Thrombin dilution	Incubation time (hr)									
	No enzyme added					Fibrinolysin added				
	0	1	3.5	21	46	0	1	3.5	21	46
1:2	5	5	5	5	5	5	5	5	5	5
1:4	6	6	6	6	6	6	6	6	6	6
1:8	8	8	8	9	9	8	8	8	9	8
1:16	13	13	13	13	14	12	13	13	13	13
1:32	20	20	21	22	22	20	22	22	24	25
1:64	30	35	39	42	46	30	34	38	42	44
1:128	55	57	70	77	77	50	52	55	65	72
1:256	85	90	120	140	180	85	100	100	125	150
No thrombin added	—	—	—	—	—	—	—	—	—	—

Body of table gives clotting times in seconds.
Incubation mixture: 5 ml thrombin + 1 ml (= 1 mg) fibrinolysin.
Clotting test: 0.5 ml incubation mixture + 1.0 ml 0.15% fibrinogen.

clot broke down on gently tilting the tube.

The fibrinogen, prepared from beef blood, was obtained through the courtesy of Dr. J. D. Porsche of Armour and Company. Negative tests were obtained for the presence of prothrombin, and no lytic activity appeared when the fibrinogen was treated with an active streptokinase preparation.

Two different sources of fibrinolysin were used in these experiments. Globulin fraction III-3‡ was first used as a convenient source of the protease, although it was later found to contain appreciable amounts of free thrombin.[10] A second source of enzyme was the more highly purified fibrinolysin described by Loomis *et al.*,[6] which gave negative tests for thrombic activity. This was used in a buffered saline solution containing 1 mg of the dry preparation per ml.

Results. Table I presents the results of an experiment in which the stability of thrombin was studied in the presence of globulin fraction III-3. The clotting times were found to remain fairly constant with the more concentrated thrombin solutions; but dilute solutions showed some increase in clotting time. However, there were no significant differences in the rate of increase in the clotting times between the controls and the samples containing added protease.[8] The samples to which globulin fraction III-3 had been added showed some additional

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‡ Obtained through the courtesy of Dr. John T. Edsall, and prepared under a contract between the OSRD and Harvard University from human blood collected by the American Red Cross.

TABLE III.
Fibrinolytic Activity of Globulin Fraction III-3 After Incubation with Thrombin at 38°C.

Thrombin dilution	Incubation time (hr)		
	0	1	2
1:1	7′	17′	53′
1:2	6′	13′	46′
1:4	6′	12′	46′
1:8	5′	23′	>4°
No thrombin added	5′	4°38′	>4°

Body of table gives lytic times in hours (°) and minutes (′).

Incubation mixture: 2 ml thrombin + 2 ml (= 2 mg) globulin III-3.

Fibrinolysis test: 0.5 ml incubation mixture + 1 ml saline + 0.2 ml 1:10 thrombin + 1 ml 0.6% fibrinogen.

thrombic activity due to the presence of a small amount of thrombin in this fraction.[10]

Table II shows the results of a similar experiment carried out with the fibrinolysin preparation. Here again it is evident that the stability of thrombin was not affected by the presence of fibrinolysin. All of the clots obtained at the 21-hour testing period were completely lysed within 24 hours; and those obtained at the 46-hour testing period were completely lysed within 54 hours, showing that active fibrinolysin was still present. However, the mixtures containing the highest concentrations of thrombin retained the greatest amount of fibrinolytic activity. The shortest lytic time (after the 46-hour incubation period) was 4 hours for the 1:2 dilution of thrombin; 9 hours for the next higher dilution; and progressively longer lytic times were observed up to a maximum of 54 hours for the sample which contained no thrombin. These observations were checked using globulin fraction III-3 in place of the fibrinolysin preparation, and the results of these lytic time measurements are given in Table III. It can be seen that the solutions containing the higher concentration of thrombin retained their fibrinolytic activity better than samples with little or no thrombin present.

Summary. It has been shown that fibrinolysin does not affect the clotting activity of thrombin, and that the thrombin preparation increased the stability of fibrinolysin under the conditions of these experiments.

15824 P

Effect of Iodine and Adrenalin on Thyrotropin in Graves' Disease and in Normal and Thyroidectomized Dogs.

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This study deals with (1) the evaluation of the amount of circulating thyrotropic factor in clinical hyperthyroidism, (2) the effect of iodine on such circulating thyrotropic factor, and (3) the influence of the parenteral injection of adrenalin on thyrotropic discharge from the anterior pituitary in the intact and the totally thyroidectomized experimental animal.

Method. Biological assay was used for the determination of thyrotropic factor. This technic involves histological examination of the thyroids of young guinea pigs not exceeding 200 g in weight, following subcutaneous injection of 5 cc of serum from the patient or experimental animal. The guinea pigs were injected on 2 successive days and were killed with ether 24 hours after the last injection. The thyroid lobes were then removed, fixed in 10% formalin, and stained with hematoxylin-eosin. The thyroids were then examined for hyperplastic changes. Following the injection of varying amounts of pure thyrotropic factor there occur changes in the thyroid characterized by a decrease in the amount of colloid in the follicles, an increase in the height of the cells lining the acini, a decrease in the size of the alveoli, and not infrequently mitosis may be observed.[1–4] These findings are not dissimilar to those observed in the thyroids of patients with Graves' disease. The amount of circulating thyrotropic factor in the serum of our patients and experimental animals was determined on the basis of the criteria just outlined.

Specimens of blood were obtained from patients with Graves' disease before treatment and again on the 2nd, 4th, 6th, 8th, and 11th days after lugolization was started. Five cc of the serum was injected into the guinea pigs on 2 successive days. The animals were killed 24 hours later and their thyroids promptly removed. On several patients samples of blood were also obtained on the 2nd, 4th, and 6th days, after subtotal thyroidectomy. The animal experimental studies were conducted in dogs. Normal dogs were injected with 1 cc of adrenalin-in-oil twice daily. On the 4th day of therapy one lobe of the thyroid was removed for histologic study. The injections were continued and the second lobe of the thyroid was removed 10 days later. In another group of animals a control sample of blood was obtained, a total thyroidectomy performed, and injections of 1 cc of adrenalin-in-oil twice daily were then begun. Specimens of blood were obtained on the 2nd, 4th, 6th, 8th, and 10th days after thyroidectomy. Five cc of serum was injected into the guinea pigs on 2 successive days and the guinea pigs' thyroids were removed 24 hours later.

Results. Thirteen patients with Graves' disease were studied in this fashion. In all these patients prior to treatment there was less circulating thyrotropic factor than is found to be present in normal individuals. This is consistent with the observation previously reported by Rawson and Starr.[4] Following lugolization there occurred an increase in circulating thyrotropic factor in all patients, which reached a peak between the 4th and 6th days and then began to diminish. In 3 instances the increase in circulating thyrotropic factor was marked, as evidenced by the profound hyperplastic changes observed in the guinea pig thyroids. In the remaining 10 patients the increase in circulating thyrotropic factor varied from slight to moderate. In a control series of 6 normal individuals who were given Lugol's solution and studied in an identical manner there occurred a barely perceptible increase in circulating thyrotropin. After subtotal thyroidectomy in patients with Graves' disease there occurred a further slight increase in the thyrotropic factor in the blood. This confirms the observations of Rawson and Starr,[4] who noted a similar although much more marked increase after *total* thyroidectomy.

Following the injection of adrenalin-in-oil in the intact dogs there occurred marked hyperplastic changes in the thyroid lobe removed on the 4th day of injection. The further administration of adrenalin-in-oil resulted in a considerable decrease in the hyperplasia of the remaining lobe which was removed 10 days later.

In the totally thyroidectomized dogs the injection of adrenalin-in-oil resulted in a marked increase in circulating thyrotropic factor, which reached its peak approximately 4 to 6 days after the beginning of treatment and thereafter began to diminish.

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15825

Recovery of the Leukocytosis-Promoting Factor in Exudates of Rabbits.*

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Earlier studies have demonstrated the presence of a leukocytosis-promoting factor in inflammatory exudates.[1–5] This factor, which has been abbreviated as the LPF has been found in canine exudates and human exudates. Canine material has been found to be active in guinea pigs and in human beings, and the factor present in human exudates was found to be active on dogs.

The factor has not been successfully isolated from rabbit exudate although its presence has been demonstrated by the author,[6] and has been confirmed by Reifenstein and his collaborators.[7] Nevertheless attempts to extract the factor from rabbit exudates resulted in failure. The rabbit is well known to be somewhat atypical as to the type of polymorphonuclear cells it contains in exudates, the type of intracellular enzymes, and the changes in the hydrogen ion concentration of the exudate, with the progress of inflammation. In the present communication observations have been collected which indicate that the factor can be extracted from rabbit exudates. Its effect on the absolute white count is not very striking when a study of normal variations is made on rabbits. However, when one studies the types of leukocytes which are being discharged as a result of the injection of the LPF, it is clear that the leukocytosis-promoting factor operates primarily in inducing a rise in the percentage of immature leukocytes. It is interesting in this connection that Danzer[8] showed that the injection in rabbits of various extracts of organs deflects the polymorphonuclear count. The extracts were prepared from normal muscle, liver, brain, and testis by shaking the minced tissue in saline. Ponder and MacLeod[9] demonstrated that the repeated injections of an irritant into the peritoneal cavity is correlated with a showering into the blood stream of very young polymorphonuclear leukocytes of Class I.

The method employed to extract the LPF from rabbit exudates can be outlined as follows:

1. Inflammatory exudates are recovered from the pleural cavity by injection of 0.2 cc of 5% croton oil in olive oil. A day or 2 later variable amounts of exudates are obtained from such region.
2. Such exudates are immediately treated with benzene in a proportion of 1:1.
3. The mixture of benzene and exudate is stirred under a hood with a stirring apparatus for a period of about 20 minutes.
4. The well-mixed contents are poured into a separatory funnel and held in such a container until good separation occurs.
5. The benzene-free fraction is now treated with ammonium sulfate at half saturation.
6. The precipitate is centrifuged. The supernatant fluid is discarded and the precipitate is dialyzed until free of sulphate ions. It is then injected in variable amounts into the marginal vein of a normal rabbit. Counts

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TABLE I.
Effect of Leukocytosis-promoting Factor of Rabbit Exudates on Number of Circulating Leukocytes.

Rabbit No.	Amt of LPF injected, cc	Basal white cell count, per mm³	Basal No. of immature WBC (one lobe), %	Max. No. of white cells within 6 hr following injection of LPF, per mm³	Max. No. of immature WBC (one lobe), %
1-53	5	9,325	2	13,950	18
1-73	22	9,600	6	17,500	38
1-59	13	8,875	2	15,400	38
19-77	20	6,850	—	16,300	30
19-73	6	10,975	4	15,400	30
1-78	9	7,600	10	21,600	35
	Avg	8,871	4.8	16,692	31.5

TABLE II.
Effect of Saline and Other Inert Material on Number of Circulating Leukocytes in the Rabbit.

Rabbit No.	Amt of material inj. cc	Basal white cell count, per mm³	Basal No. of immature WBC (one lobe), %	Maximum No. of white cells within 6 hr after injection, per mm³	Maximum No. of immature WBC (one lobe), %
2-5	5 Saline	8.400	4	14,850	4
19-92	5 ’’	7,825	—	9,750	—
2-5	5 ’’	6,350	—	17,400	—
18-24	6 Serum (rabbit)	5,350	0	9,250	2
19-97	15 Saline	6,625	—	11,550	—
1-82	5 Benzene in horse serum	8,100	—	13,650	—
1-88	10 Saline	9,075	4	16,100	14
19-73	20 ’’	8,225	4	12,150	2
1-61	8 Horse serum	9,325	4	13,400	10
	Avg	7,697	3.2	13,122	6.4

are made every hour for several hours and blood smears are likewise taken.

The results of such a series of experiments appear in Table I. It is clear that although the rise in the circulating leukocytes is not excessive, yet the percentage of immature leukocytes is markedly increased as a result of such an injection.

The results obtained when the normal variation in the leukocytes of rabbits is studied indicate that the striking effect of the LPF is primarily on the differential count. The same fails to hold when various inert materials are injected into the circulating blood of normal rabbits. The results appear in Table II.

Summary. The leukocytosis-promoting factor is present in rabbit exudates, and it has been extracted from such fluids. The extracted LPF has primarily an effect in inducing the discharge of immature leukocytes. The effect on the absolute level of leukocytes is to increase it, but this effect is not pronounced.

15826

Susceptibility of the Gray Fox to Fox Encephalitis.

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Studies on the adaptation of the virus of fox encephalitis to animal species have shown that this virus can invade related groups of animals with varying degrees of pathogenicity. In members of the family Canidae, such as the red and silver foxes, dogs, and coyotes,[1] experimental infection with the virus results in a high mortality, while such species as the black bear[2] and the raccoon,[3] which are offshoots of the canines, are only slightly susceptible.

The gray fox (Urocyon) is only distantly related to the red fox group (Vulpes). The latter is related to European forms, whereas the gray fox is probably a survivor of American preglacial fauna and appears to be more closely related to the South American foxes and dogs. In view of these relationships, it has been of interest to study the susceptibility of gray foxes to fox encephalitis.

Our earliest experiments[1] indicated that gray foxes inoculated with the virus by cisterna puncture did not show evidence of infection. In a later experiment one of 2 gray foxes inoculated by simultaneous cisterna puncture and intramuscular injections developed symptoms of fox encephalitis and was killed at the end of 4 days. Microscopic examination revealed characteristic inclusion bodies in the meninges and brain capillary endothelium. Inclusions were also seen in hepatic and reticular-endothelial cells of the liver and in the endothelium of the kidney glomeruli. More recently we have been able to show that a symptomless infection can be produced regularly in gray foxes and that only occasionally will an infection result in death.

Attempts were made to infect gray foxes by intracerebral, intraperitoneal, or intraocular injections. Such inoculations in red foxes result in an acute systemic infection with a high mortality.[4,5] Endothelial cells of organs infected, particularly the endothelial cells of the brain capillaries, show intranuclear inclusion bodies which are characteristic of the disease.[6] When the virus is inoculated into the anterior chamber of the eye, the single layer of endothelial cells lining the posterior surface of the cornea is infected. This is indicated by the presence of inclusions and the development of a corneal opacity.[5]

The virus regularly used in our experimental work is a homogenized suspension of infected fox brain preserved as 20% tissue in 50% neutral glycerin. The source of each of virus lots 77, 88, and 98 were pooled brain suspensions from not less than 10 infected red foxes which were allowed to die from the infection or were killed *in extremis*. These pooled viruses were subsequently tested in red foxes and were found to be highly virulent.

In a preliminary experiment the 20% suspension of pooled lots 77, 88, and 98 was diluted to a 2% suspension in Ringer's solution. 0.1 cc was injected into the anterior chamber of the left eye of an 8-weeks-old gray fox pup. In 3 days the eye was completely opaque. The animal was killed, and smears of the corneal endothelium showed typical intranuclear inclusions. The aqueous humor was bacteriologically sterile. No inclusion bodies were observed in the brain capillaries. Presence of the virus in the brain of the gray fox was shown by aseptically removing a portion of the cerebellum, which was then ground with mortar and pestle and diluted to a 5% suspension with Ringer's solution. 0.1 cc was injected into the anterior chamber of the eye of the highly susceptible red fox, which developed a corneal opacity in 4 days and died of typical fox encephalitis in 5 days.

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0.25 cc of 20% pooled virus from lots 77, 88, and 98 was then inoculated intracerebrally into two 8-weeks-old gray fox pups. At the end of 5 days these animals had not developed symptoms, although with red foxes a 2- to 4-day incubation period was commonly observed under similar conditions. The gray foxes were killed and their brains and livers were removed aseptically. Microscopic examination revealed no evidence of infection. Equal parts of the brains were pooled and the mixture diluted to 20% in Ringer's solution. This was designated "gray fox passage virus" and was inoculated into a second group of 4 young gray fox pups as follows: (1) 0.25 cc intracerebrally and 0.1 intraocularly into the anterior chamber of the left eye: (2) 0.25 cc intracerebrally; (3) and (4) 0.25 cc intracerebrally simultaneously with 0.25 cc of pooled 20% virus from lots 77, 88, and 98.

The first gray fox, which was inoculated with gray fox passage virus intracerebrally and intraocularly, developed a slight corneal opacity in 3 days. The opacity became progressively marked by the end of 8 days, at which time the animal appeared sluggish in its movements and would not eat. The fox was killed, and inclusions were found in the corneal endothelium but not in the brain or other tissues. A portion of the brain was removed aseptically, ground and diluted to a 5% suspension in Ringer's solution, and injected into the anterior chamber of the eye of a red fox. This animal developed a positive eye reaction in 3 days and died in 5 days with symptoms and microscopic findings of fox encephalitis. The second gray fox, inoculated with gray fox passage virus intracerebrally, was normal at the end of 5 weeks.

The 3rd and 4th gray foxes were inoculated intracerebrally with gray fox passage virus together with equal amounts of original red fox virus. One animal remained normal, while the other developed symptoms of fox encephalitis by the 8th day. This animal had occasional convulsions and exhibited a marked weakness of its hind legs. When standing up, it appeared to be in a lethargic state, but weaved back and forth unsteadily. The fox was killed *in extremis*. Numerous intranuclear inclusions were found in the brain capillary endothelium and in the liver reticular-endothelium. Material from the brain of this animal, subsequently injected intraocularly into a red fox, produced the typical eye reaction, symptoms, and death, with typical pathology of fox encephalitis.

The 2 gray foxes that remained normal in this group developed a distemper infection 5 weeks later and were discarded.

Pooled brain and liver from all the gray foxes that showed evidence of a fox encephalitis infection, together with the original pooled virulent red fox virus from lots 77, 88, and 98, were inoculated into a group of 4 young gray foxes. 0.5 cc of a 20% suspension of the pooled tissues was injected intracerebrally, simultaneously with 2 cc injected intraperitoneally. Two of the animals were sacrificed at the end of 6 days, although no symptoms had been observed. Microscopic examination of the brains of both animals revealed several typical intranuclear inclusions in the capillary endothelium. The brain material, subsequently inoculated intraocularly into red foxes, produced the characteristic eye reactions, symptoms, and death from fox encephalitis. The 2 gray foxes that were allowed to live remained normal for 5 weeks, after which time they developed a distemper infection and were discarded. When brain from one of these animals was inoculated intraocularly into a red fox, no viable virus was found.

Summary. The results indicate that the gray fox is relatively resistant to fox encephalitis virus virulent for red foxes. It appears that the virus will regularly infect the gray fox to produce a symptomless infection, but only occasionally will symptoms appear that resemble the disease in red foxes.

A virulence gradient of this virus for related species can be postulated whereby in the family *Canidae* the closely related dogs and coyotes (*Canis*) and red foxes (*Vulpes*) are most susceptible, the more distantly related gray fox (*Urocyon*) less susceptible, and those species which are earlier offshoots of the canines, such as the black bear and the raccoon, are the least susceptible to fox encephalitis.

15827

Viability of the Rabbit Papilloma Virus.

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Rabbit papillomatosis was described by Shope,[1] who established that the tumorous growth was due to a filterable virus. The occurrence of the rabbit papilloma seems to have been first recorded by Seton,[2] but the warty growths generally described as rabbit horns were well known even previously to hunters and naturalists. Thaddeus Surber[3] noted the growths on Kansas cottontail rabbits in 1899.

Our first studies of rabbit papillomatosis were in 1927 and since samples of rabbit horns collected by Green at that time were preserved, it is now possible to test samples for viability that have been in storage for almost 20 years. Recently we have tested a group of 6 viruses in storage from approximately 6 to 20 years.

From the reports in the literature, it has become obvious that stored papilloma tissue would remain viable for long periods of time. Shope in his original work used papilloma tissue which had been stored 106 days. Bryan and Beard[4] used papilloma tissue which had been stored for 3 years and found it to be capable of producing infections. Samples of the Shope virus in our laboratories have been stored as pieces of papillomatous tissue in 50% glycerine, refrigerated at 4°C.

The tissues were tested for viable virus by scarifying small areas of skin on the ears and flanks of domestic rabbits and applying small amounts of centrifuged tissue suspensions. In preparing material for inoculation, tissues were ground with a mortar and pestle and suspended in 9/10% saline to give a tissue suspension of about 10%. The suspension was centrifuged for one-half hour at 2000 r.p.m. The supernatant was used for the skin inoculation and was applied with a

TABLE I.
Duration of Viability of Shope Papilloma Virus.

Tissue No.	Source of tissue	Date collected	Date tested	Length of proven viability
1	Cottontail rabbit	Mar. 1927	Jan. 1947	19 years 10 mo.
2	” ”	Jul. 1937	Nov. 1946	9 ” 4 ”
3	” ”	Apr. 1938	Jun. 1946	8 ” 2 ”
4	” ”	Feb. 1939	Jun. 1946	7 ” 4 ”
5	” ”	Mar. 1939	Jul. 1946	7 ” 4 ”
6	” ”	Apr. 1940	Sep. 1946	6 ” 5 ”

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[4] Bryan, W. R., and Beard, J. W., *J. Nat. Cancer Inst.*, 1941, **1**, 607.

cotton swab. The dates of virus collection and testing are given in Table I.

All of the above tissues proved capable of producing typical confluent papillomatous growths in domestic rabbits. The incubation period in each case was approximately 2 weeks except for tissue No. 1, for which the incubation period was about 3½ weeks.

Summary. Cottontail papilloma tissue stored in 50% glycerine at 4°C remains infective and capable of producing confluent papillomatosis for as long as 20 years. All 6 of the tissues tested after storage from 6 to 20 years were found viable. This seems to establish that Shope virus is extremely stable when stored under refrigeration.

15828

A Pithed Rat Preparation Suitable for Assaying Pressor Substances.

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Although pithed cats and pithed dogs are very useful as sensitive test preparations for studying or assaying pressor substances, the cost for procurement and maintenance of the animals is a considerable disadvantage. Because rats are more readily available and are much more economical to use when large numbers of test animals are involved, an effort was made to develop a pithed rat preparation which would satisfy the requirements for pressor sensitivity and reproducibility. In the present paper a technic is described for setting up a satisfactory rat preparation which compares favorably with, and in several respects is superior to, the pithed cat or dog.

Young male rats (albino or hooded) weighing 150-300 g were used. The rat was anesthetized by the intraperitoneal injection of sodium amytal, 0.09 mg/g body weight. Atropine sulfate (1.2 mg) was given along with the anesthetic. The trachea was exposed and a piece of plastic tubing or rubber catheter 6 cm long and 2.5 mm in diameter was inserted into the opened trachea. Tubing of this size fitted snugly and did not need to be tied in place.

Either the femoral or common carotid artery was isolated for cannulation. A short cannula of small diameter (0.5 mm) was tied in the artery and connected to a mercury manometer by plastic or rubber tubing. A dilute solution of heparin was used to prevent clotting in the cannula. (It is advisable to use a small bore [3.0-3.5 mm] mercury manometer to minimize the loss of blood from the rat by displacement into the manometer during rises in blood pressure.)

The vagi were then cut and the jugular veins and carotid arteries tied. With the rat on its back and the hind feet pinned to the operating board the animal was ready to be pithed. The pithing rod, 2.2 mm in diameter, 25 cm long, with one end bluntly pointed, was made from an ordinary wire coat hanger. By holding the rat's head taut and in line with the vertebrae, with the thumb in the angle of the mandible and the forefinger around the top of the skull, the point of the pithing rod was inserted obliquely into and through the eye socket at an angle of approximately 45° to the long axis of the rat. After the skull was entered the rod was realigned with the vertebral column and passed through the cranium and thence down the whole length of the spinal canal. The pithing rod was left in place on the assumption that it would afford mechanical compression of blood vessels torn by the rod in the process of pithing.

The tracheal cannula tubing was then promptly connected to the respirator system which included a side tube fitted with an adjustable screw clamp. The respirator used was an air-driven, windshield wiper type with adjustments for varying both rate and

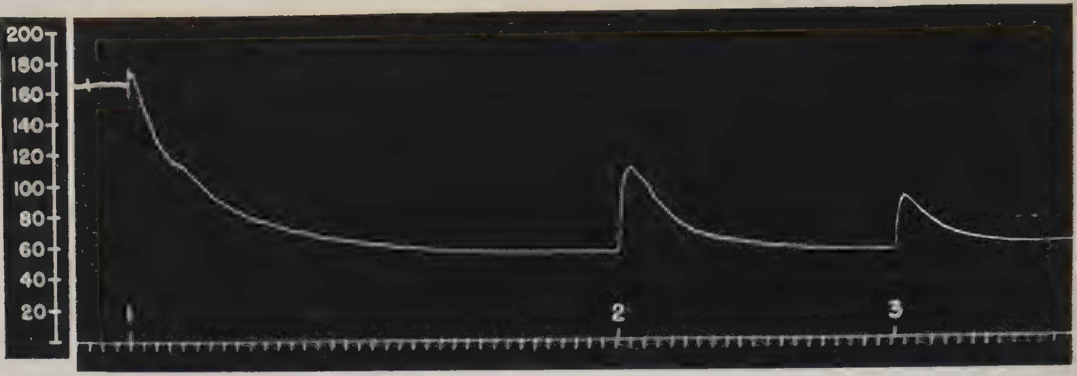

FIG. 1.

Record of mean blood pressure in a pithed rat preparation. Wt. 180 g. 1. Rat pithed. 2. I.V. injection of 6 μg of a "laboratory standard" angiotonin powder in 0.2 ml saline. 3. I.V. injection of 0.1 μg epinephrine hydrochloride in 0.2 ml saline. Ordinate scale—blood pressure in mm Hg. Time marker—1 minute. (32ZD-14.)

volume. (Great care must be taken to avoid hyperventilation or overdistention of the lungs if the development of pulmonary edema is to be prevented.) Respiratory rates of 50-60 per min. were found to be optimum for the rat. Slower rates of 30-40 were tolerated but oxygenation of the blood appeared to be inadequate. With rates slower than 20 the rats lived for only a short time.

For making intravenous injections a small portion of skin was cut away from the area overlying the femoral vein. Into one end of a 6-inch piece of very small plastic tubing (1 mm O.D.)* was fitted the shaft of a 24-gauge hypodermic needle which had been cut off below the syringe hub. The needle was inserted into the femoral vein and left in place, the application of a ligature being unnecessary. Syringes of 0.25 or 1.0 ml capacity and graduated in 0.01 ml were fitted with 26- or 24-gauge hypodermic needles the beveled points of which had been ground off flat. Usually 0.1 to 0.2 ml of test sample was given, syringe and needle removed from the plastic tube and replaced by a second syringe and needle with 0.1 ml physiological saline wash. Care had to be taken to avoid the inclusion of very small air bubbles when changing syringes. This could be accomplished by injecting 0.02 ml of saline and quickly replacing syringe and needle with the test sample syringe and needle. During this exchange a slight outward flow of saline would expel the small air bubble usually present as the result of the transfer.

The animal was left undisturbed for ½ hour before injections were begun during which time the depth of respiration was observed and altered if necessary according to the color of the arterial blood and the extent of movement of the thorax. With a little practice it was found that the optimum exchange of air could be gauged with reasonable accuracy from the excursions of the chest wall.

Thirty to 40 minutes after the rat was pithed the mean blood pressure usually leveled off between 40 and 60 mm Hg. Pressor responses of 30-40 mm Hg were obtained in most of the preparations with only 0.05-0.1 μg of epinephrine. Occasionally comparable rises in blood pressure were obtained with as little as 0.0125 μg. In general, the sensitivity to angiotonin was from 2 to 5 times as great as that exhibited by the pithed cat. A sample record of typical pressure curves is shown in Fig. 1.

With proper maintenance of the depth and rate of artificial respiration, satisfactory pressor responses were obtained for periods averaging 5 to 7 hours. Over 120 pithed rats have been prepared by the method described and approximately 90% of these have been satisfactory test animals. In the majority of instances technical errors or the use of rats which showed signs of illness were thought to be responsible for preparations which exhibited poor pressor responses or died prematurely.

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* "V-1" plastic tubing, A. C. Balfour Associates, Englewood, N.J.

Summary. A simple method is described for setting up a sensitive pithed rat preparation suitable for assaying small quantities of pressor substances.

Grateful acknowledgment is made to Mr. C. Wilson whose technical experience in preparing pithed cats has been a valuable aid in the present study.

15829

Effect of Pentaquine in Patients with Hypertension.*

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The recent observation that pentaquine [6-methoxy-8-(5'-isopropylaminoamylamino)-quinoline], a new antimalarial agent, produces postural hypotension in normal man[1,4] raised the question of its usefulness in treating hypertensive patients. The purpose of the present report is to summarize briefly the results of a clinical trial of this compound in a random group of 17 patients with long standing essential hypertension including 3 in the malignant phase of the disease.

Procedure and Results. Pentaquine was administered orally in amounts varying from 120 to 240 mg of the base per day, given in equally divided doses every 4 hours. Arterial pressure was measured in the arm by the standard auscultatory method using a mercury manometer. Measurements were made with the patient resting comfortably in the supine position, and also, during and after 5 minutes (or less if syncope intervened) of motionless standing. Mean arterial pressure was calculated as one-half the sum of the systolic and diastolic blood pressure. The pulse rate was palpated at the wrist or auscultated at the cardiac apex. Patients were hospitalized for at least 3 days prior to treatment, and were maintained on essentially the same regimen before, during and after the period of drug therapy.

In the majority of cases (Table I), after 2 to 7 days of treatment at dosages of 120 to 240 mg per day, a reduction of systolic and diastolic blood pressure occurred, varying from 10 to 40% of the mean arterial blood pressure. At the same time there was usually a further fall in blood pressure in the erect position associated with narrowing of pulse pressure. The pulse rate remained unchanged, or actually decreased in the supine position, and in the majority of patients failed to rise abnormally in the upright position even during marked hypotension.

The development of postural hypotension was not observed in all cases. Four patients exhibited a definite fall in resting blood pressure without the development of a further hypotension when erect. Others maintained a lower resting blood pressure for several weeks after the disappearance of the postural effect. However, one patient exhibited a reduction of blood pressure only in the upright position. In a few patients the depression of arterial pressure first appeared several days after the medication had been discontinued because of toxic reactions.

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* This investigation was supported in part by the Squibb Institute for Medical Research, New Brunswick, N.J. The pentaquine used was supplied by James A. Shannon, M.D., of the Squibb Institute.

[1] Loeb, R. F., *J. A. M. A.*, 1946, **132**, 321.

[4] Craige, B., Jr., Jones, R., Eichelberger, L., Alving, A., Pullman, R. N., and Whorton, C. M., to be published.

TABLE I.
Summary of Cases.

Sex	Age	Blood pressure prior to treatment	Dosage pentaquine, mg/day	Supine blood pressure at time of max. fall	Days to return to pretreatment blood pressure	Postural hypotension	Toxic effects
F.	50	220/120	120-2 days 180-3 ”	150/90	12+	+++	++++
M.	41	210/130	180-2 ” 240-1 ”	180/118	2	+	+++
F.	36	240/140	120-3 ”	Discontinued	Severe vomiting and pain		
F.	48	180/110	120-3 ” 180-4 ”	140/90	3+	+	++
M.	46	200/100	180-2 ” 240-2 ”	No response			++
F.	46	230/130	120-2 ” 180-3 ”	125/88	6	++++	+++
F.	41	230/130	120-3 ” 180-3 ”	150/100	20	++	++
F.	41	220/120	120-14 ”	No response		Agranulocytosis	
F.	46	170/110	120-2 ”	140/90	20+	++	+
M.	34	170/120	120-2 ” 180-4 ” 240-4 ”	135/110	7+	0	++
F.	46	220/140	120-4 ” 60-6 ”	180/120	4	0	++
F.	49	240/150	120-3 ” 180-4 ”	240/150	Postural hypotension 5 days		+
M.	51	200/130	120-3 ” 90-7 ”	170/90	30+	0	+
F.	49	260/135	120-2 ” 180-4 ” 60-11 ”	190/100	15	0	++
M.	47	250/170	120-3 ”	140/70	Terminal uremia		
F.	34	240/140	90-11 ”	140/90	20	+++	++
M.	45	230/160	120-6 ” 120-2 ”	160/100	12	+++	+++

The last 3 patients were in the malignant phase of essential hypertension.

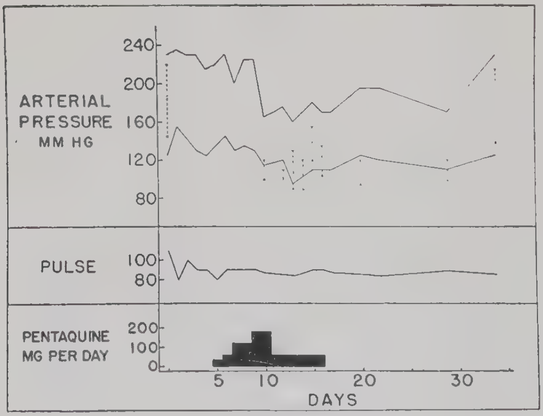

FIG. 1.

Typical response to pentaquine. The vertical broken lines represent arterial pressure readings after 2 minutes in the upright position.

The effective dose varied markedly, one patient requiring 120 mg per day given for 2 days, while several failed to respond to doses as high as 240 mg given for 5 days. Fig. 1 illustrates the typical response of a patient with essential hypertension.

The lowering of blood pressure was often preceded and accompanied by abdominal pain and tenderness, back and chest pain, frequently girdle in character, facial pallor, anorexia, nausea and vomiting, constipation or diarrhea, loss of weight in a few patients, and rarely by fever. Nausea and vomiting and/or abdominal pain were occasionally sufficiently severe to necessitate discontinuation of treatment. Impotence was noted in 2 male patients. Methemoglobinemia and moderate hemolytic anemia occurred in earlier cases but were later successfully controlled by the simultaneous administration of 1 grain of methylene blue with each dose of pentaquine. Cyanosis without signs of cardiac failure or significant methemoglobinemia appeared in a few patients.

In addition to these toxic effects, agranulocytosis developed in one patient after 2 weeks of continuous therapy, and prolonged menses appeared in another, accompanied by evidence of increased capillary fragility. Both patients recovered uneventfully following cessation of therapy and treatment with penicillin in the case of agranulocytosis. There were no other severe reactions in the group.

Following cessation of full doses of the drug the blood pressure gradually returned to its previous level over a period of several days to several weeks. In most cases the hypotensive effect was not prolonged by the administration of 50 to 60 mg of pentaquine per day. The depression of blood pressure and postural hypotension recurred, however, when the drug was again administered in full doses.

The patients with a malignant phase of essential hypertension as manifested by neuroretinitis, impairment of renal function, high diastolic blood pressure, weight loss and other signs of rapidly progressing disease, responded to pentaquine similarly as patients with uncomplicated essential hypertension, except that they uniformly required lower dosage (120 mg per day). Coincident with the fall in blood pressure there was considerable regression of the pathological changes in the fundi, relief of headaches and cessation of gross hematuria, but apparently no improvement in renal function as measured by clinical and clearance tests.

Hemodynamic studies using Hamilton manometers for recording arterial pressure and the ballistocardiograph for measuring cardiac output, before and after the hypotensive effect had been achieved, indicated that pentaquine caused a reduction of sympathetic vasopressor reflexes similar to that occurring after surgical sympathectomy.[2] This lack of responsiveness was most apparent in those cases which exhibited marked postural hypotension. Measurements of the skin temperature in the extremities under control conditions also indicated a depression of sympathetic activity.[3] Pressor responses to epinephrine and ephedrine were unimpaired, but epinephrine did not prevent collapse in the erect position. Marked postural hypotension and collapse were prevented, however, by the use of a tight abdominal belt similar to that worn following lumbodorsal splanchnicectomy. There were no consistent changes in either the electrocardiogram or in the cardiac output.

Discussion. The most interesting effect obtained with pentaquine in hypertensive patients resembled that found in certain normal subjects,[4] namely the appearance of postural hypotension. In addition, hypertensive patients often exhibited a reduction in resting blood pressure. The frequent occurrence of toxic symptoms at first suggested that the hypotensive effect was merely a reflection of toxic debility. However, there was no uniform relation between the appearance of other toxic symptoms and of the hypotensive effect. Furthermore, the hypotension was frequently maintained for several weeks after the disappearance of all other toxic symptoms, and in such instances was associated with considerable subjective improvement. These results indicate that while the drug is too toxic in the dosage necessary to produce a fall in blood pressure, its toxic and hypotensive qualities may not be inseparable.

The preliminary studies suggested that the mode of action of pentaquine upon the vasomotor system is to depress sympathetic nervous reflexes. Its activity differed from that of certain other sympatholytic agents such as the tetraethylammonium salts in that the depression of blood pressure was more prolonged and was not accompanied by a marked quickening of pulse rate. This maintenance of a normal heart rate indicates that its depressor effects include the cardiac accelerator mechanisms. That pentaquine did not block the reactivity of the vascular system to pressor agents was shown by the continued effectiveness of epinephrine and ephedrine. It is of interest that Moe and Seevers[5] reported central impairment of sympathetic reflexes in dogs treated with plasmochin, a closely related 8-aminoquinoline.

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[2] Wilkins, R. W., and Culbertson, J. W., unpublished data.

[3] Mixter, G., Jr., and Freis, E. D., unpublished data.

Summary. 1. The administration of pentaquine in high dosage to patients with essential hypertension frequently produced a significant reduction in resting arterial blood pressure, usually accompanied by the development of postural hypotension. This depressor effect occurred abruptly after several days of therapy and receded gradually over a period of several days to several weeks following cessation of therapy.

2. Patients with malignant hypertension exhibited a similar response, but did not require as high dosage. With the fall in blood pressure there was some regression of neuroretinitis, headache and gross hematuria: but no significant improvement in renal function.

3. Pentaquine appeared to exert its effects primarily through a sympatholytic action.

4. Toxic reactions to the drug were too frequent and severe to consider its use practicable in the treatment of essential hypertension.

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[5] Moe, G. K., and Seevers, M. H., *Fed. Proc.*, 1946, **5**, 193.

15830

Hemagglutination with Newcastle Disease Virus (NDV).

ALFRED L. FLORMAN.* (Introduced by F. L. Horsfall, Jr.)

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Newcastle disease of fowl, an acute infection associated with severe intestinal, respiratory and nervous symptoms, was first recognized as a distinct entity by Kraneveld[1] in the Dutch East Indies and by Doyle[2] in Newcastle, England. Doyle gave the disease its present name and demonstrated that the causative agent is filterable.

Recently there has been increased interest in the virus which causes Newcastle disease, especially because of its capacity to cause hemagglutination.[3] In addition, although the western hemisphere was thought to be free of the disease, Beach[4] showed that there is an immunological similarity between classical Newcastle disease virus (NDV) and the virus of avian pneumoencephalitis, a disease which is of frequent occurrence in this country. The potential human pathogenicity of the virus for man was indicated in 1943 with the report of an accidental laboratory infection.[5] This resulted in a brief, mild illness associated with conjunctivitis and general symptoms. In 1946 2 similar laboratory infections[6] and a small epidemic of conjunctivitis among women who had been exposed to infected fowls were reported.[7]

Burnet[3] has raised the possibility of "an affinity between NDV and the influenza virus group" chiefly on the basis of hemagglutinating capacity, infectivity for the chick embryo and apparent particle size. However, no immunological relationship could be demonstrated, and recently Bang[8] and others[9] have shown by electron microscopy that NDV

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* Aided by a grant from the Welt Fund of Mt. Sinai Hospital, New York.

[1] Kraneveld, F. C., *Nederl. Indisch. Blad. Diergeneesk*, 1926, **38**, 448.

[2] Doyle, T. M., *J. Comp. Path. and Therap.*, 1927, **40**, 144.

[3] Burnet, F. M., *Austral. J. Exp. Biol. and Med. Sci.*, 1942, **20**, 81.

[4] Beach, J. R., *Science*, 1944, **100**, 361.

[5] Burnet, F. M., *Med. J. Austral.*, 1943, **2**, 313.

[6] Anderson, S. G., *Med. J. Austral.*, 1946, **1**, 371.

[7] Yatom, J., *J. A. M. A.*, 1946, **132**, 169.

[8] Bang, F. B., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 5.

[9] Cunha, R., Weil, M. L., Beard, D., Taylor, A. R., Sharp, D. G., and Beard, J. W., *J. Immunol.*, 1947, **55**, 69.

TABLE I.
Successive Titrations of Newcastle Disease Virus at Room Temperature.*

No. of days chicken red cells were stored†	Dilution of virus‡												End point
	4	8	16	32	64	128	256	512	1024	2048	4096	8192	
1	1	2	2	2	2	2	2	2	2	0	0	0	1024
2	1	2	2	3	3	3	3	3	3	2	±	0	2048
3	1	1	2	1	1	2	3	2	2	2	1	0	2048
4	±	1	2	3	3	3	3	3	3	2	1	0	2048

* Room temperature = 22°C to 27°C.
† Red cells obtained from one chicken, and stored at 4°C, concentration = 0.75%.
‡ Reciprocal of final dilution of pool 3.
Results were read at 30 minutes.

TABLE II.
Successive Titrations of Newcastle Disease Virus at 4°C.

No. of days chicken red cells were stored*	Dilution of virus†											End point
	4	8	16	32	64	128	256	512	1024	2048	4096	
1	4	4	4	4	4	4	3	2	1	±	0	512
2	4	4	4	4	4	4	4	4	2	1	0	1024
3	4	4	4	4	4	4	3	2	1	0	0	512
4	4	4	4	4	4	4	4	3	2	±	0	1024

* Red cells, identical to Table I.
† Reciprocal of final dilution of pool 3.
Results were read at 60 minutes.

is strikingly different from the influenza viruses.

In a study of the phenomenon of hemagglutination, NDV was investigated. It was found that the methods which have been developed for influenza viruses were not satisfactory with NDV. When hemagglutination titrations were carried out at room temperature, there was much less marked agglutination with low virus dilutions than with higher ones. Even with more dilute chicken red cell suspensions and a shorter reaction time as recommended by Brandly *et al.*[10] this disturbing effect persisted and the reactions were difficult to read and to reproduce. It is the purpose of this communication to show how this effect can be circumvented by carrying out hemagglutination reactions with NDV in the cold and to offer an explanation for this peculiar property of the virus. In addition, some of the quantitative aspects of hemagglutination by NDV will be presented.

Virus. A strain of Newcastle disease virus (NDV) was obtained from Dr. G. K. Hirst who had received it from Dr. F. R. Beaudette. Further passages were carried out by inoculation into the allantoic fluid of 9-12-day-old chick embryos. After approximately 48 hours incubation at 37°C, the eggs were refrigerated at 4°C for 3-18 hours, after which the allantoic fluid was collected. Infected fluid was stored either at –70°C or at 4°C.

Sera. An authentic antiNDV serum was obtained from the Bureau of Animal Industry, U. S. Department of Agriculture, Washington, D. C. Specific antisera were prepared in rabbits by slowly injecting into the ear vein 10 cc of allantoic fluid containing the virus. A second intravenous injection, identical to the first, was given after 6 weeks and serum was obtained 2 weeks later.

Saline. An 0.85% solution of sodium chloride containing 0.01M phosphate buffer at pH 7.2 was used throughout.

Chicken red cells. Blood was obtained from the wing vein of individual chickens in an excess of 2% sodium citrate. The cells were washed twice in the usual manner, and suspensions of desired concentration were prepared in saline from cells which had been packed by 10 minutes centrifugation at 1500 r.p.m.

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[10] Brandly, C. A., Moses, H. E., Jungherr, E. L., and Jones, E. E., *Am. J. Vet. Research*, 1946, **7**, 289.

TABLE III.
Effect of Temperature on Titrations with Newcastle Disease and Influenza Viruses.

Temp.,°C	RBC 1.5%	Virus	Dilution of serum† 8	16	32	64	128	256	512	1024	2048	End point
22	chicken	NDV	0	±	1	1	2	3	3	1	0	512
4	”	”	4	4	4	3	3	2	±	0	0	256
22	”	PR8	4	4	4	4	4	4	4	2	1	1024
4	”	”	4	4	4	4	4	4	3	3	0	1024
22	”	Lee	4	4	4	4	4	4	3	1	±	512
4	”	”	4	4	4	4	4	4	3	±	0	512
22	human	NDV	1	2	2	3	3	3	2	0	0	512
4	”	”	2	1	±	0	0	0	0	0	0	8
22	”	PR8	4	4	3	3	3	2	2	1	0	512
4	”	”	4	4	3	3	2	2	2	0	0	512
22	”	Lee	4	4	3	3	3	3	1	0	0	256
4	”	”	4	4	3	3	2	0	0	0	0	128

* Reciprocal of final dilution.
Results were read at 60 minutes.

Test tubes. Pyrex tubes 75 by 10 mm were found to be satisfactory.

Titration of virus. As was stated above, when a titration of NDV is carried out by the hemagglutination technic at room temperature, an effect which simulates a serological prozone appears. This is evident from the results shown in Table I. However, if the reaction is carried out in cold (4°C), as originally suggested by Burnet,[3] this effect disappears and clear cut end-points are obtained, as can be seen from the results shown in Table II. Therefore, the following titration technic was employed: Serial 2-fold dilutions of infected allantoic fluid are made in cold saline. To each 0.4 cc of dilution an equal volume of chilled 1.5% chicken red cell suspension is added. The tubes are shaken and immediately placed in the refrigerator (4°C) for one hour. The patterns of sedimented cells are then noted while the tubes are still cold. Complete agglutination is considered to have occurred if a solid plaque of cells, which does not stream on tilting the tube, is seen. It is recorded as 4. Partial agglutination is represented by an even thin deposit of cells on the bowl of the tube, 3; a similar cell pattern with a small central mass of unagglutinated cells, 2; and by a larger central mass with only a few clumps of agglutinated cells along its edge, 1. The end-point is taken as the highest dilution in which a 2 reaction occurs.

When virus titrations are carried out in the cold, the end-point obtained with NDV is usually lower by 2 to 4 times than when the test is performed at room temperature. (Compare the results in Tables I and II). However, the pattern of hemagglutination and titration end-points obtained in the cold is sufficiently reproducible to permit a reasonably accurate estimate of the dilution which corresponds to one agglutinating unit (*i.e.*, the highest dilution giving a 2 reaction). This estimate is of importance in agglutination-inhibition tests for antibodies against the virus.

Care in the selection of a source of red cells for hemagglutination tests with NDV is important. Although human Group O erythrocytes are agglutinated by NDV, they give much poorer reactions in the cold than at room temperature. Results similar to those shown in Table III were obtained with red cells from each of 3 persons; the end-points were approximately 64 times lower at 4°C than at 22-27°C. Erythrocytes from occasional individual chickens are also markedly insensitive to agglutination by the virus in the cold. By selection, however, chickens can be obtained, the red cells from which give end-points not more than 2 times lower at 4°C than at room temperature. Erythrocytes from such chickens are employed as a routine. Chicken red cell suspensions are stored at 4°C and may be used for at least 4 days without appreciably changing the results of titrations, as is shown in Table II.

The weak agglutination seen with low dilu-

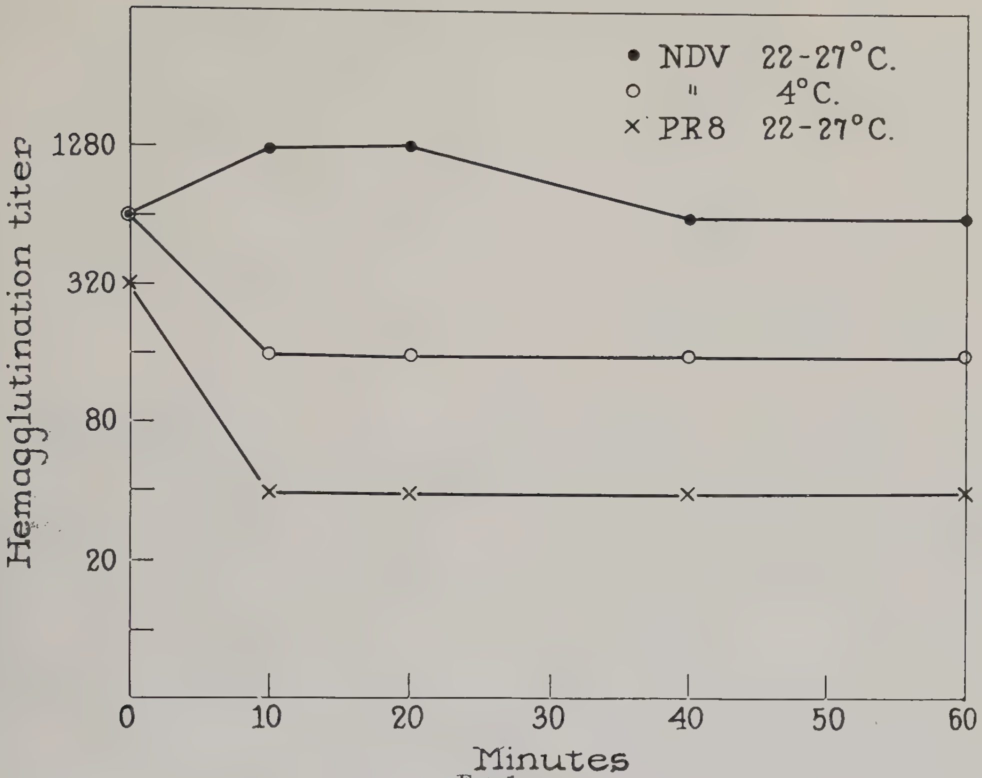

FIG. 1.
Adsorption of NDV and PR8 by chicken red cells. Hemagglutination titers of supernatant at various intervals after mixing red cells and virus.

tions of NDV at room temperature as compared to the complete agglutination observed at 4°C suggested that the difference might be dependent upon the rates of adsorption and elution of NDV from red cells at these temperatures. Experiments were carried out to determine this at 4°C and at room temperature. Equal volumes of 3% chicken red cell suspensions and NDV-infected allantoic fluid were mixed. At intervals aliquots were removed and immediately centrifuged. The supernates were tested for the presence of residual virus by the hemagglutination method. For comparison a similar experiment was performed with influenza A virus at room temperature. The results of these experiments are presented graphically in Fig. 1. Although no evidence was obtained that adsorption of NDV by red cells occurred at room temperature, it seems probable that it actually occurs but is masked because of very rapid elution of the virus from erythrocytes. Adsorption of NDV by red cells can be demonstrated when the mixture is held at 4°C and at that temperature elution of NDV from erythrocytes does not occur for at least 60 minutes. Hirst[11] has shown that temperature influences the rate of elution of influenza viruses from erythrocytes in a similar manner. It appears probable that the difference noted when NDV is titrated at room temperature and at 4°C is dependent on the elution of virus which occurs at room temperature[3] with the result that agglutination is masked in lower dilutions of virus. However, Cunha and his associates[9] have recently reported the presence in allantoic fluid of a substance which interferes with red cell agglutination by NDV.

Hemagglutination caused by NDV may be simply and readily differentiated from that produced by influenza viruses on the basis of the weak agglutination which is obtained with both chicken and human red cells at

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[11] Hirst, G. K., *J. Exp. Med.*, 1942, **76**, 195.

TABLE IV.
Agglutination-inhibition Tests with NDV and Antiserum.

Preliminary incubation of serum-virus mixtures*	Rabbit serum	Dilution of serum†									End point
		20	40	80	160	320	640	1280	2560	5120	
None	Normal	3	2	3	4	4	4	4	4	4	
	Anti-NDV	±	0	±	0	1	4	4	4	4	1:480
1 hr at 4°C	Normal	4	4	4	4	4	4	4	4	4	
	Anti-NDV	0	0	0	0	0	2	4	4	4	1:640
1 " " 37°C	Normal	4	4	4	4	4	4	4	4	4	
	Anti-NDV	0	0	0	0	0	0	0	4	4	1:1920

* Virus sufficient to yield 8 agglutinating units in the final mixture was employed.
† Reciprocal of final serum dilution.
Results were read after 60 minutes at 4°C.

room temperature with low dilutions of NDV, as well as by the relative failure of human red cells to show agglutination with NDV in the cold. These differences are clearly evident in the experiments summarized in Table III in which parallel titrations of NDV, PR8 and Lee viruses were made with the same suspensions of chicken and human red cells. Allantoic fluid infected with NDV usually gives hemagglutination titers ranging from 1:320 to 1:512, whereas 50% infectivity end-points as determined in chick embryos are of the order of 10^{-10}. This indicates a difference between hemagglutination and virus titers with NDV which is 10 to 100 times greater than that usually obtained with influenza viruses.[12–14]

Titration of antibody. Because of the distinct advantages of performing hemagglutination tests with NDV in the cold, the usual method for measuring antibodies against the virus by means of agglutination-inhibition was modified as follows: The virus is titrated in the cold with the same chicken red cell suspension that is to be used in the agglutination-inhibition test. The final dilution of allantoic fluid which is equivalent to 32 agglutinating units is determined, prepared, and its actual titer checked by a second hemagglutination test. The sera to be tested are heated at 56°C for 30 minutes and diluted in 2-fold series. To each tube containing 0.2 cc of serum dilution, 0.2 cc of virus (diluted so as to equal 32 units) is added. The mixtures are shaken and incubated for 60 minutes in a water bath at 37°C. They are then cooled to 4°C and 0.4 cc of cold 1.5% chicken red cell suspension is added to each tube. After 60 minutes at 4°C the tests are read and the lowest serum dilution showing 2 plus, or greater, agglutination is taken as the end-point.

The results of parallel agglutination-inhibition tests with antiNDV serum are presented in Table IV. The conditions of preliminary incubation of the serum-virus mixtures were varied as indicated and the advantage of incubation at 37°C for one hour before the addition of red cells is clearly demonstrated. This modification was originally suggested by Burnet.[5]

To determine the reproducibility of serum dilution end-points (*i.e.* antibody titers) obtained by means of the agglutination-inhibition method described above, the following experiment was performed: Sera from each of 4 rabbits which had been immunized against NDV were tested separately against 3 different amounts of virus (*i.e.*, 32, 16 and 8 units, respectively). The results are shown graphically in Fig. 2. The end-points obtained were closely similar with each of the 4 immune sera and a constant amount of virus. Moreover, as the quantity of virus used was *increased*, the serum dilution end-point *decreased* proportionately. These results indicate that the reaction between NDV and specific antibody *in vitro* shows a quantita-

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[12] Taylor, A. R., Sharp, D. G., Beard, D., Beard, J. W., Dingle, J. H., and Feller, A. E., *J. Immunol.*, 1943, **47**, 261.

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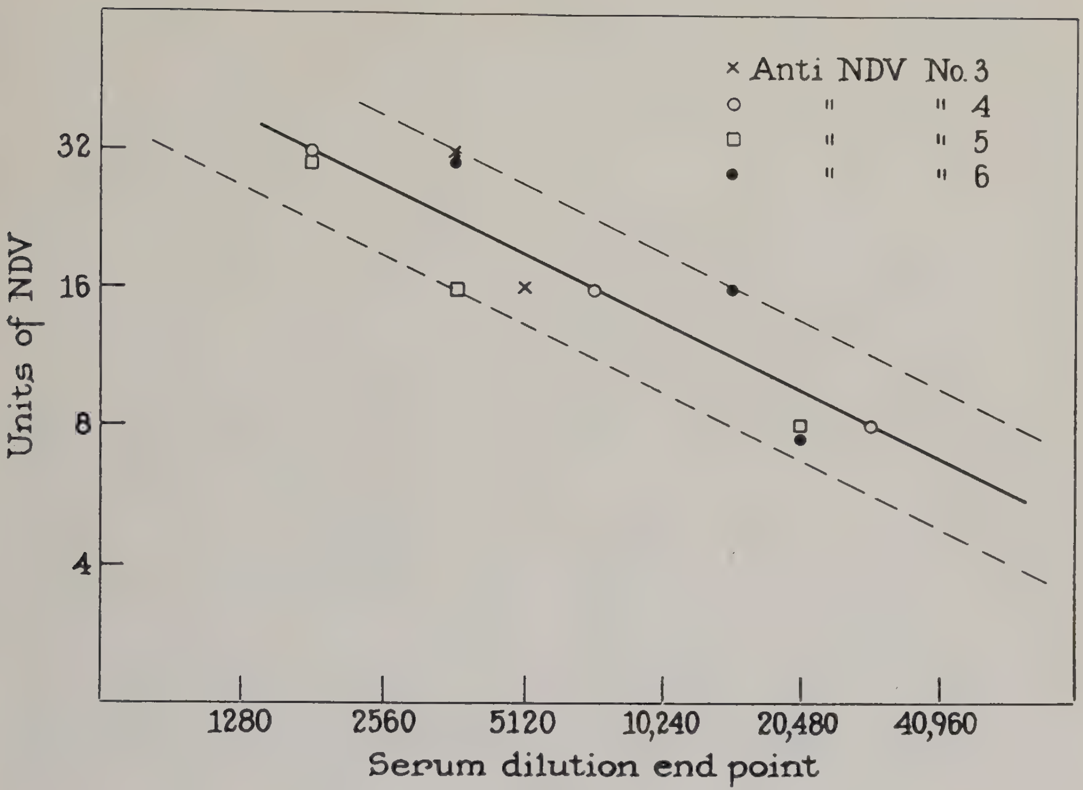

FIG. 2.

Distribution of serum dilution end points in agglutination-inhibition tests with varying amounts of NDV. Antisera from 4 rabbits immunized against NDV. Dotted lines represent the limits of twofold deviations (a single tube) from the solid line.

tive relationship analogous to that previously demonstrated with certain other viruses [*i.e.*, influenza and pneumonia virus of mice (PVM)] as well as with classical antigen-antibody reactions.

Summary. Hemagglutination by Newcastle disease virus (NDV) is markedly affected by temperature. At room temperature the reaction is difficult to read, especially with low dilutions of virus. At 4°C the reaction is easily read and titration end-points are readily determined.

At 4°C NDV appears to be adsorbed more completely by and to elute less rapidly from chicken red cells than at room temperature.

Details of satisfactory methods for titration of virus and antibodies against it are presented. Both tests are carried out at 4°C.

15831

Effect of Ergotamine Tartrate on Potassium Changes in Histamine Shock.*

CAROLINE TUM SUDEN. (Introduced by H. O. Haterius.)

From the Department of Physiology, Boston University School of Medicine.

Serum potassium changes in histamine shock were investigated in rats treated with ergotamine tartrate (ET-rat) and compared with the previous findings on intact rats and rats with autoplastic grafts of adreno-cortical tissue (ACT-rat)[1] as an approach to the question of the relative importance of medullary and adrenocortical factors concerned with the release of potassium in histamine shock.

Methods. Serum potassium was determined for adult male rats (Long-Evans strain) at 30 and 60 minutes following intraperitoneal injections of histamine phosphate† (10 mg/100 g body weight). Parenteral administration of ergotamine tartrate‡ (0.5 mg/100 g body weight) was given one-half hour prior to the injections of histamine. The dosage of ergotamine used was sufficient to depress adrenergic activity, as determined by epinephrine reversal, for the strain used. Details of operative procedure and chemical methods of analysis were identical with those previously described.[1]

Results. Experiments to date have led to the following observations on the rat treated with ergotamine:

1. Some reduction of the normal fluctuation of serum potassium was indicated by the decrease in the standard deviation.

6 cases, average 21.3±0.8 mg/100 ml
cf. controls 21.3±1.3 mg/100 ml

2. No significant effect upon the rising trend of serum potassium during development of histamine shock (30-minute values) was obtained.

5 cases, average 24.0±1.9 mg/100 ml
cf. controls, 24.5±1.7 mg/100 ml

3. A possible accentuation in fall of serum potassium occurred during subsidence of histamine shock (60-minute values).

5 cases, average 19.8±2.5 mg/100 ml
cf. controls 22.8±3.5 mg/100 ml

4. A possible exacerbation of the rise in serum potassium ensued upon repeated or extensive bleeding (see 6, below).

3 cases, average 27.9±0.26 mg/100 ml
cf. controls 23.8±1.4 mg/100 ml

The determinations were made on a second blood specimen of 4 ml taken immediately after the first 4 ml bleeding.

5. An elevation of serum potassium followed upon higher dosages of histamine. After 20 and 40 mg histamine in single paired experiments the values obtained 60 minutes later were respectively:

26.8 mg/100 ml and 30.6 mg/100 ml for the ET-rats;
cf. 24.4 mg/100 ml and 24.0 mg/100 ml for controls.

6. No marked influence on gross symptoms was found. Atonia appeared slightly aggravated; under anesthesia respiratory failure upon bleeding was more easily induced than in the controls. The higher values for serum potassium as found in (4) and (5) above correlated with this observation. Toxic reactions from ergotamine alone were not especially apparent in the rat and were in no way as severe as reported for the dog (2).

Some observations were made on rats bearing grafts of adrenocortical tissue and similarly treated with ergotamine. Blood samples were analyzed for potassium at 30 minutes (3 cases) and at 60 minutes (4 cases) following the injection of histamine (10 mg/100 g). The potassium averages for the 2 groups were comparable to those obtained in histamine-shocked rats with only regenerated cortex: *e.g.*

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* This investigation has been made with the assistance of a grant from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry, American Medical Association.

[1] tum-Suden, C., Wyman, L. C., and Derow, M. A., *Am. J. Physiol.*, 1945, **144**, 102.

† Burroughs Wellcome and Company.

‡ Gynergen, kindly supplied by Sandoz Chemical Works, Inc.

35.5±0.5 mg/100 ml at 30 min., ergotamine;
34.2±5.8 ” ” ” ” ” , no ergotamine;
41.0±2.4 ” ” ” 60 ” , ergotamine;
38.5±6.5 ” ” ” ” ” , no ergotamine;

The ergotamine-treated preparations differed from the nontreated in showing less variability in potassium levels. Survival, as judged both by time and number, seemed slightly better than expected for this class of animal receiving a dosage of histamine (10 mg) which previously was fatal to 60% of the nontreated within one hour.

Discussion. The data obtained indicated that fluctuations in serum K tended to be suppressed by ergotamine tartrate. In the dosage used the difference from the untreated controls was not significant. However, if maximum amounts of ergotamine had been employed a slight lowering of the potassium levels might have been established as found by Houssay for the dog.[2]

Symptomatically, the course of histamine shock following upon the administration of 10 mg intraperitoneally appeared unchanged both in the intact rat and the rat bearing adrenocortical grafts. There was no evidence of a potentiation or synergism between cortical hormones and epinephrine as might be surmised from the findings of Vogt that the latter prolongs cortin secretion.[3] The lack of such interaction therefore cannot account to any extent for the high susceptibility in rats possessing only adrenocortical tissue. The experiments serve to emphasize the inefficiency of regenerated cortical substance, the relation of the rise in extracellular potassium to level of cortical activity and the minor influence of the sympathico-adrenal mechanisms upon potassium release in histamine shock.

Summary. Ergotamine tartrate in the dosage used did not significantly modify serum potassium changes characteristic of histamine shock in the rat. It did not influence histamine tolerance in either the normal or the demedullated animal.

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15832

Effect of Sex Hormones on Pituitary Lactogen and Crop Glands of Common Pigeons.*

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Estrogens, testosterone propionate and progesterone can exert a dynamic stimulus to the secretion of lactogenic hormone by the anterior pituitary. This is particularly true of the estrogens which have been shown to increase the pituitary lactogen content of rats,[1-3] guinea pigs[3,4] and rabbits[5] from 200 to 500%. A 40% increase has been obtained with testosterone propionate in rats.[6] Progesterone apparently has no effect on the lactogen content of the A.P. when administered in small or moderate dosages, but amounts well above the physiological level in rats (15.0 mg per day for 10 days) have increased it about 74% on a body weight basis.[7] Selye has shown that many steroid hormones can exhibit common "folliculoid" properties when administered in sufficient dosages,[8] and this may well account for the action of progesterone on hypophyseal lactogen. From a cytological point of view it is interesting to note that estrogens and testosterone propionate have been shown to increase the number and secretory activity of the eosinophilic cells of the anterior pituitary,[9–13] which are agreed to be the source of lactogenic hormone in both mammals[14–16] and pigeons.[17] The ability of sex hormones, particularly estrogens, to increase lactogen in the anterior pituitary is also reflected in the greater quantities of lactogenic hormone found in the blood of rabbits after estrone administration[5] and in the initiation of milk secretion in a number of mammals following estrogen administration.[4,18] The authors have advanced the theory that estrogen is the factor normally responsible for stimulating the secretion of pituitary lactogen and initiating milk secretion at the time of parturition.[4]

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* Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series No. 1040.

[1] Reece, R. P., and Turner, C. W., *Mo. Agr. Exp. Sta. Res. Bull.* No. 266, 1937.

[2] Lewis, A. A., and Turner, C. W., Proc. Soc. Exp. Biol. and Med., 1941, **48**, 439.

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[4] Meites, J., and Turner, C. W., *Endocrinology*, 1942, **30**, 711.

[5] Meites, J., and Turner, C. W., Proc. Soc. Exp. Biol. and Med., 1942, **49**, 190.

[6] Reece, R. P., and Mixner, J. P., Proc. Soc. Exp. Biol. and Med., 1939, **40**, 66.

Crop secretion in pigeons is analagous to milk secretion in mammals and both processes furnish the means for supplying nutrition to the young during the critical period after birth. Would the sex hormones, therefore, stimulate pituitary lactogenic secretion in pigeons as in mammals? Furthermore, if these steroids could increase the lactogen content of the pigeon pituitary, would it also be reflected in the initiation of crop gland secretion?

Experimental. Groups of 16 to 20 mature pigeons of both sexes were injected subcutaneously over the pectoral muscles daily for a 10-day period. These birds were kept together in groups of 8 to 10 in a cage. All the sex hormones were dissolved in corn oil and injected in 0.1 cc volumes. On the day after the last injection the birds were killed, weighed, sexed, and the pituitaries and crop glands were removed. The crop glands were washed clean, freed from adhering fat tissue, blotted dry between towel papers and weighed. They were then examined under light for evidence of visual proliferation. In the case of the diethylstilbestrol group and its controls, which constitute a preliminary experiment performed in 1942, the crop glands were not weighed but only examined for visual proliferation.

The pituitaries of each group were macerated with a small mortar and pestle, suspended in a definite volume of distilled water and injected intradermally over the crop glands of 10 common pigeons in 0.1 cc volumes for 4 days. The equivalent of 6 male or 4 female pigeon pituitaries was given to 10 birds, and each group of pigeon pituitaries was directly compared with another by injecting over both sides of the crop gland. The crop glands were rated for lactogenic response by the Reece-Turner method.[1]

Results. It will be seen that in the preliminary experiment performed in 1942, 250 μg of diethylstilbestrol did not increase the lactogen content of the pituitary or initiate crop milk secretion in 13 female pigeons. In the 1946 series, total dosages of 1,000 I.U. estrone, 10 mg progesterone or 5 mg testosterone propionate† caused no significant

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† We are indebted to Dr. D. F. Green of Merck & Co., Rahway, N.J., for the diethylstilbestrol; to Dr. D. W. MacCorquodale of The Abbott Research Labs., North Chicago, Ill., for the estrone; and to Dr. F. E. Houghton of Ciba Pharmaceutical Products, Inc., Summit, N.J., for the progesterone and testosterone propionate used in this study.

change in the lactogen content of the pituitary, and there was no evidence of visual proliferation or weight increase in the crop glands. The differences in the pituitary lactogen content between the 1942 and 1946 female control pigeons may be due to the fact that the former pigeon pituitaries were assayed in the early fall while the latter were assayed in mid-winter. As in a previous report on the lactogen content in the pituitaries of common pigeons,[19] it was found that the female pituitary contains considerably more lactogen than the male. None of the sex hormones increased the pituitary weight of the pigeons.

Discussion. There appears to be an inverse relationship between the secretion of gonadal hormones and the formation of crop milk in pigeons. Thus, it has been demonstrated that (1) the testes and ovaries decrease in size during incubation and brooding, when crop secretion normally occurs,[20-22] and (2) the administration of lactogenic hormone markedly reduces the size of the pigeon gonads.[23,24] With the exception of one report in which nonhormonal factors favorable to broodiness were introduced,[25] the injection of sex hormones has proven ineffective in stimulating crop secretion in pigeons.[26,27] The mechanism responsible for the secretion and release of lactogen from the pigeon hypophysis may depend chiefly on psychic and

TABLE I. Effect of Sex Hormones on Pituitary Prolactin and Crop Glands of Common Pigeons.

Treatment	Sex	Total No. of pigeons	Avg body wt, g	Avg pituitary wt, mg	Avg crop wt, g	Avg crop wt / 100 g body wt	No. of visual crop responses	Avg No. lactogen units* per pit.	Avg No. lactogen units / 100 g body wt	Avg No. lactogen units / mg pit. tissue
		1942								
Controls	F	8	276	4.32			0	2.00	.72	.46
250 Diethylstilbestrol	F	13	292	4.13			0	2.31	.79	.56
		1946								
Controls	1F	8	311	3.71	2.20	.70	0	1.62	.52	.43
	3M	10	326	3.44	2.47	.76	0	.85	.26	.25
5 mg Testosterone propionate	2F	11	285	3.82	2.22	.77	0	1.75	.69	.46
	4M	6	311	3.20	2.23	.71	0	1.12	.36	.35
10 mg Progesterone	2F	9	284	3.32	2.11	.74	0	1.50	.53	.45
	3M	7	326	3.28	2.52	.77	0	.85	.26	.25
1000 I.U. Estrone	1F	10	295	3.61	1.79	.60	0	2.06	.69	.54
	4M	7	336	3.45	2.22	.66	0	1.21	.36	.35

* Reece-Turner lactogen units 22.2 R.T. units = 1 International Unit (Meites, J., Bergman, A. J., and Turner, C. W., *Endocrinology*, 1941, **28**, 707).

1,2,3,4 These groups were compared with each other for lactogenic hormone by injecting their pituitaries over both sides of the crop glands in the same 10 pigeons.

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environmental rather than hormonal stimuli.[26] It seems clear from our study that the injection of sex hormones alone are ineffective in augmenting lactogen output by the pituitary or initiating crop milk secretion in pigeons.

It appears remarkable that the pigeon pituitary should be refractory to the administration of sex hormones, particularly estrogens which are such powerful activators of lactogenic hormone secretion in the pituitaries of mammals. Neither do estrogens increase the weight of the pigeon pituitary as in mammals. It would be of interest to determine what effect the sex hormones have on the cytology of the pigeon hypophysis.

Summary. The effects of estrone, diethylstilbestrol, progesterone and testosterone-propionate were determined on the lactogen content and weight of the pituitary, and on the proliferation and weight of the crop glands of common pigeons of both sexes. The results were all negative. It is concluded that the pigeon pituitary, unlike the mammalian, is refractory to the administration of sex hormones and is not stimulated by them to increase the secretion of lactogenic hormone.

15833

Statistical Evaluation of Growth Curves.

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A problem that frequently confronts research workers in the biological sciences is the comparison of growth curves of groups of experimental animals subjected to different treatments. The customary practice has been that of visual estimation of significant differences from a plot of weight against time (Fig. 1), or the application of the "t" test to the mean weights at a particular time. These conventional methods of evaluation often give an ambiguous or unconvincing analysis of the results.

The purpose of this paper is to demonstrate how time-weight data (growth curves) have been treated more critically in a comparative study of exposure of rats to vapors

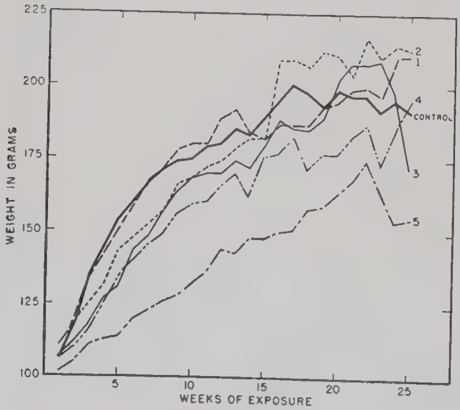

Fig. 1.
Combined growth curves of male and female rats.

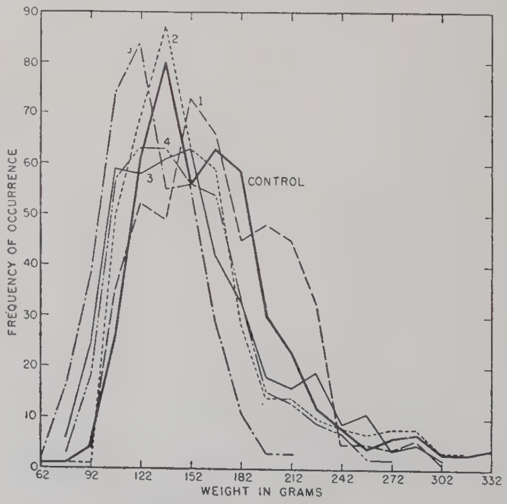

Fig. 2.
Curves showing the frequency of occurrence of individual weights for male and female rats, throughout the period of treatment.

TABLE I.
Summary of Data Showing Differences in Weight Response.

Group	No. of rat wts	Mean wt	Standard dev.	''t'' at 12 wks exposure	Probability level for 12 wk ''t''	$3_{\sigma}d/d$	Chi square where $\chi^2 = \Sigma \left(\frac{(fo - ft)^2}{ft}\right)$	Probability level for chi square
Combined Weights of Male and Female Rats.								
1	464	168.4	40.6	0.72	0.50	3.74	19.8	0.14
Control	451	166.1	45.8	—	—	—	—	—
2	444	157.3	47.7	0.44	0.65	1.07	16.8	0.27
3	429	154.0	46.0	0.72	0.50	0.77*	31.1	0.008*
4	395	147.7	36.9	1.03	0.30	0.46*	31.4	0.002*
5	371	127.4	25.4	2.94	0.005*	0.20*	126.6	<0.001*
Weights of Male Rats.								
1	220	195.2	48.8	0.05	0.95	2.08	7.6	0.66
Control	209	188.0	54.5	—	—	—	—	—
3	227	169.2	54.8	0.54	0.60	0.84*	17.3	0.24
2	234	167.3	53.0	0.64	0.54	0.74*	9.7	0.70
4	180	157.1	44.2	1.03	0.32	0.49*	24.0	0.013*
5	200	133.0	28.1	2.77	0.014*	0.23*	72.5	<0.001*
Weights of Female Rats.								
1	244	158.0	31.4	1.74	0.11	0.66†	21.4	0.003†
Control	242	146.6	23.6	—	—	—	—	—
4	215	140.0	27.1	0.12	0.91	1.09	9.8	0.20
3	202	137.1	24.0	0.35	0.73	0.72*	14.8	0.01*
2	210	135.1	20.0	0.82	0.43	0.53*	17.6	0.004*
5	171	119.4	25.6	2.18	0.04*	0.27*	57.6	<0.001*

* Decrease from control group is statistically significant.
† Increase above ''   ''   ''   ''   ''

of a series of 5 analogous halogenated hydrocarbons. Each group consisted of about 30 animals, with males and females in approximately equal numbers; the sexes being kept separately. These groups were exposed on alternate days for 25 weeks, and each animal was weighed weekly. A control group was exposed to air alone. Fig. 1 shows the combined growth curves of the male and female rats as normally plotted, with time the abscissa and weight the ordinate. With the exception of Group 5, it is impossible to designate which group does or does not vary significantly from the control. The "t" test was applied to the weights of the 12th week of exposure as at this time the majority of the original animals were still alive. Again, with the exception of Group 5, nothing of significance was found. As a solution to the problem, the weight data were expressed in the form of frequency distributions and the weight curves were compared as one treats normal curves of other biological data. Each individual weekly weight of each rat alive at the time of weighing was considered a point in the distribution. Separate frequency distributions were prepared for the male and the female rats. These data were combined to show the overall effect of the materials. Fig. 2 illustrates the shape of these curves, where weight is the abscissa and the frequency of occurrence of each weight is the ordinate.

With the data in the form of frequency distributions, statistical comparison of the curves is simple and straight-forward. The method of chi square reveals similar probability levels of significance, as does the ratio of 3 times the standard error of the difference, to the difference between the mean weights. In each case exposed rats were compared to controls. A summary of the chi square and "t" data is presented in Table I. The coefficient of skewness was calculated for the distributions of the control groups, and was found to be 0.48 for the combined male and female group, 0.19 for the male and 0.55 for the female controls. These values justify the use of normal statistics.

Discussion. The use of chi square is more valid for the comparison of weight-response curves than is a test of differences between means. In the chi square test the frequency with which a given weight range occurs in the treated group is directly compared to the frequency with which the same weight range occurs in the control group and there is made no arbitrary selection of a particular period for weight evaluation as is done in the "t" test. It was necessary to combine some weight classes at the extremities of distributions where the rats did not attain the spread in range of weights of the controls and where the theoretical frequencies of the classes were less than 5.

The use of a frequency distribution, in which each individual weekly weight is a point, yields a sufficient amount of valid data to make a good comparison of the weights of comparatively small groups of animals. In Table I the values for the groups of animals are listed in decreasing order of the mean weights. A remarkable relationship may be seen between the rank of the groups by this method of listing and by the magnitude of chi square and the $3\sigma d/d$ ratio.

Summary. By the application of frequency distributions to growth data, familiar statistical comparisons may be applied to determine whether significant differences exist. Previously no valid method was available to compare adequately growth curves in their entirety. The use of the chi square test on the distributions is deemed the method of preference for showing such differences.

15834

Effect of Insulin Hypoglycemia and Glucose on Various Amino Acids in Blood of Mental Patients.

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In previous publications[1,2] we reported that the level in the blood of the total amino acids and also of glutamine is markedly depressed during the insulin hypoglycemic shock treatment of mental patients. It was also shown that the administration of glucose[2] produces a similar effect although it is not so marked as that produced during insulin hypoglycemic shock.

As part of an investigation regarding the metabolism of various amino acids in mental patients, determinations were made of the effect of insulin and of the administration of glucose on the level in the blood of the following amino acids: arginine, histidine, leucine, lysine, phenylalanine, tryptophane and valine.

It is known that the metabolism of some specific amino acids may be disturbed in certain clinical conditions as for example, phenylalanine in oligophrenia phenylpyruvica.[3,4] Gjessing[5] and others have reported on alterations in nitrogen metabolism in certain types of periodic psychotic episodes. In some pathological states certain amino acids may serve some special functions as for example, methionine in the protection of the liver against some poisons.[6,7] Furthermore it is known that some amino acids can be synthesized in the animal organism in amounts adequate for its needs while others are essential and must be supplied in the diet for normal nutrition.[8]

No study regarding a group of individual amino acids has thus far been carried out in mental patients owing to the lack of adequate methods. The recent development of microbiological methods has made such study feasible.

Procedure and Methods. A group of 15 patients undergoing or about to undergo a course of insulin hypoglycemic shock treatment was used for this study. The patients were about 16 hours postabsorptive when the observations were begun. They were kept in bed during the entire period of study.

After a preliminary specimen of venous blood was taken, the patient was given either a dose of insulin subcutaneously which produced hypoglycemic coma, or 75 g of glucose in 200 ml of tap water which was flavored with lemon juice. Nothing was administered to 4 patients who were used as controls.

Blood was withdrawn from the anterior cubital vein in an oiled syringe and discharged into a flask containing heparin. The blood samples were taken to the laboratory immediately and centrifuged for 10 minutes and the plasma separated. The plasma was again centrifuged for 5 minutes to remove any stray red blood cells. The plasma proteins were then immediately precipitated with tungstic acid according to the method of Hier and Bergeim.[9] The precipitate was removed by centrifugation and filtration. The filtrate was adjusted to pH 7 with 1N NaOH using the Beckman pH meter.

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[3] Penrose, L. S., and Quastel, J. H., *Bioch. J.*, 1937, **31**, 266.

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[7] Miller, L. L., and Whipple, G. H., *J. Exp. Med.*, 1942, **76**, 421.

[8] Sahyun, M., *Outline of the Amino Acids and Proteins*, 1944, p. 223.

[9] Hier, S. W., and Bergeim, O., *J. Biol. Chem.*, 1945, **161**, 717.

Each determination was carried out in triplicate. Leuconostoc mesenteroides P-60 and the improved medium D of Dunn *et al.*[10] were used for the determination of lysine and histidine. Tryptophane was determined according to Dunn *et al.*[11] using *Lactobacillus arabinosis*. The medium of Hac, Snell and Williams[12] and *Lactobacillus casei* were used for the determination of arginine. Leucine and valine were determined with the medium of Shankman[13] and *Lactobacillus arabinosis*. Phenylalanine was determined with the medium of Dunn *et al.*[14] and *Lactobacillus casei*.

One milliliter of basal medium was employed in all the determinations except that of histidine. For the determination of lysine, valine and phenylalanine, 1 ml of blood filtrate was used and for arginine, tryptophane, and leucine only 0.5 ml of filtrate plus 0.5 ml of water was employed. For the determination of histidine it was found necessary to increase the volume of basal medium to 2.5 ml to which was added 1 ml of filtrate and 1.5 ml of water.

A set of standards was run with each determination instead of depending upon standard curves. This acted as a check as to any changes in the behavior of the microorganisms.

Tryptophane and histidine were determined turbidometrically with the Coleman spectrophotometer with the light band set at 540. Electrometric titration, with the Beckman pH meter, of the acid production was used for the determination of the other amino acids.

The total amino acid nitrogen was determined by the colorimetric method of Frame *et al.*[15,16]

The blood sugar was determined by the colorimetric method of Somogyi.[17]

The Coleman spectrophotometer was used for all colorimetric determinations.

Observations. Table I shows the effect of insulin hypoglycemia on the level of the total amino acid nitrogen and of various amino acids in the blood plasma. It will be noted that the total amino acids and all of the individual amino acids studied thus far, were significantly depressed during insulin hypoglycemic coma. However, the various amino acids were not depressed to the same degree. This is indicated by the marked variations in the percentage drop from the initial levels of the various amino acids in the same patient (Table I). Thus for example, in patient (J.B., No. 1) lysine is depressed 70% whereas the level of the total amino acids is depressed only 33%. It will also be seen that the levels of leucine and lysine are the 2 amino acids most markedly depressed during insulin hypoglycemic coma. The drop in the levels of leucine ranged from 63 to 81% of the initial level, and that of lysine ranged from 47 to 77%. With a few exceptions, the percentage drop in the level of the individual amino acids was equal or greater than the drop in the level of the total amino acids (Table I).

The initial level of the amino acids appeared to bear no relationship to the dose of insulin required to produce hypoglycemic coma, and the extent of the drop of the level of the amino acids was not directly related to the size of the dose of insulin which was administered.

Table II shows the effect of the administration of 75 g of glucose, orally, on the blood sugar level and the corresponding changes in the level of the total amino acids and of some of the individual amino acids in the blood plasma. The greatest drop in the level of the amino acids occurred in the first hour after the administration of glucose, at which time the level of the blood sugar was the highest. There was, with an occasional exception, a further drop at the end of 2 hours at which time the blood sugar level was either

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[10] Dunn, M. S., Shankman, S., Camien, M. N., Frankl, W., and Rockland, L. B., *J. Biol. Chem.*, 1944, **156**, 703.

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[12] Hac, L. R., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, 1945, **159**, 273.

[13] Shankman, S., *J. Biol. Chem.*, 1943, **150**, 305.

[14] Dunn, M. S., Shankman, S., and Camien, M. N., *J. Biol. Chem.*, 1945, **161**, 643.

[15] Frame, E. G., Russell, J. A., and Wilhelmi, A. E., *J. Biol. Chem.*, 1943, **149**, 255.

[16] Russell, J. A., *J. Biol. Chem.*, 1944, **156**, 467.

[17] Somogyi, M., *J. Biol. Chem.*, 1945, **160**, 69.

TABLE I.

Effect of Insulin Hypoglycemia on the Level of Various Amino Acids in the Blood Plasma.

Patient and No.	Sex	Age, yr	Wt, kg	Dose of insulin units	Total amino acid nitrogen mg/100 ml		Arginine γ/ml		Histidine γ/ml		Leucine γ/ml		Lysine γ/ml		Tryptophane γ/ml		Valine γ/ml	
					*B	*A	B	A	B	A	B	A	B	A	B	A	B	A
J.B. 1	M	29	81	220	6.4	4.3 (33)†	23.8	13.5 (43)	15.3	6.9 (55)	57.6	20.5 (64)	25.4	7.6 (70)	14.6	9.0 (38)	34.	17.4 (49)
J.M. 2	M	29	64	160	7.2	4.1 (43)	22.1	13.5 (39)	16.5	6.7 (59)	63.4	15.8 (75)	32.8	7.6 (77)	15.8	9.6 (39)	35.6	13.5 (62)
W.B. 3	M	34	66	150	6.4	4.0 (38)	21.4	12.8 (40)	12.3	7.0 (43)	52.8	19.6 (63)	29.1	15.3 (47)	11.3	9.2 (19)	45.9	27.9 (39)
J.B. 4	M	32	91	200	7.3	4.8 (34)	34.	16.2 (52)	16.4	8.0 (51)	60.6	17.0 (72)	42.	17.4 (59)	13.7	9.1 (34)	46.5	27.8 (40)
A.J. 5	M	29	72	340	6.0	3.9 (35)	23.8	11.8 (50)	13.0	7.5 (42)	52.	10.8 (79)	15.7	5.2 (67)	12.8	8.4 (34)	41.2	23.1 (44)
A.M. 6	M	23	65	130	7.2	3.8 (47)	29.6	10.8 (64)	14.3	8.1 (43)	57.	11.1 (81)	28.9	7.4 (74)	12.8	6.8 (47)	44.4	23.1 (48)
R.A. 7	F	21	72	100	8.9	4.1 (54)	25.5	8.7 (66)	13.4	6.7 (50)	62.8	20.1 (68)	36.6	11.9 (68)	21.6	14. (35)	38.2	17.8 (53)
L.S. 8	F	31	49	120	7.8	4.8 (39)	33.9	16.8 (51)	12.1	7.6 (37)	54.9	14.7 (73)	35.0	13.5 (61)	16.5	10. (39)	34.5	14.6 (58)
F.DeG. 12	M	19	91	0	6.5	6.6	30.9	28.2	12.2	13.1	63.6	57.0	26.5	26.5	14.1	13.0	44.3	40.4
R.L. 13	M	18	83	0	6.5	6.4	24.3	24.9	10.0	11.3	61.8	59.4	21.2	24.0	12.4	12.2	39.6	38.1
N.D. 14	F	18	46	0	5.9	5.8	22.0	22.2	14.4	13.7	51.9	54.9	34.5	33.5	15.1	12.7	35.7	34.5
R.L.‡ 15	M	23	51	0	5.9	5.9	24.9	24.6	13.1	13.2	49.8	48.9	21.7	23.6	10.8	10.0	31.8	27.3

* B = Level before the administration of insulin.

* A = Level 3½ hours after insulin, at which time patients were in hypoglycemic coma. Controls, without insulin, show the effect of 3½ hours rest in bed.

† = Figures in parentheses indicate the depression of the level of the amino acids in per cent of the initial level.

‡ = Phenylalanine was determined in this patient and was 7.2 γ and 7.1 γ per ml before and after 3½ hours of rest, respectively.

TABLE II.

Effect of the Oral Administration of Glucose on the Level of Various Amino Acids in the Blood Plasma.

Patient and case No.	Sex	Age yr	Wt kg	Time hr	Blood sugar mg/100 ml	Total amino acid nitrogen mg/100 ml	Arginine γ/ml	Histidine γ/ml	Leucine γ/ml	Lysine γ/ml	Phenyl-alanine γ/ml	Trypto-phane γ/ml	Valine γ/ml
S.W. 9	M	15	72	0*	92	6.1	24.4	14.2	55.8	24.8	—	14.0	33.6
				1	152	5.3 (13)†	20.4 (16)	12.2 (14)	42.0 (25)	21.3 (14)	—	11.5 (18)	26.7 (21)
				2	80	5.1 (16)	18.0 (26)	11.3 (20)	32.1 (42)	19.8 (20)	—	11.3 (19)	21.8 (35)
				3½	79	5.1 (16)	19.2 (21)	12.0 (15)	37.5 (33)	19.1 (23)	—	10.7 (24)	21.1 (37)
J.F. 10	M	20	67	0	90	6.4	29.4	12.5	63.8	30.3	6.5	12.2	45.2
				1	127	5.3 (17)	19.5 (34)	11.0 (12)	48.4 (24)	24.5 (19)	5.6 (14)	10.2 (16)	33.6 (26)
				2	109	5.4 (16)	17.7 (40)	10.4 (17)	37.8 (41)	24.5 (19)	5.1 (22)	9.2 (24)	26.7 (41)
				3½	75	5.3 (17)	17.2 (41)	11.0 (12)	38.1 (40)	25.2 (17)	4.5 (31)	10.1 (16)	25.1 (44)
C.C. 11	F	17	52	0	85	5.4	22.8	12.7	37.8	19.4	5.1	9.4	24.0
				1	138	4.8 (11)	15.6 (32)	12.2 (4)	25.2 (33)	16.1 (17)	4.5 (12)	8.5 (10)	17.5 (27)
				2	—	—	—	—	—	—	—	—	—
				3½	74	4.4 (19)	13.8 (39)	12.0 (6)	25.6 (32)	17.4 (10)	5.1 (0)	9.9 (−5)	15.0 (38)

* = Time interval after the administration of glucose.

† Figures in parentheses represent the depression of the amino acids in per cent of the initial level.

lower or higher than the initial postabsorptive level. After 3½ hours the blood sugar fell below the initial level while the amino acids all remained significantly depressed below the initial level and tended to show either a slight rise or fall from the level reached at the 2-hour period.

The findings in 4 control patients who did not receive insulin are shown at the bottom of Table I. After 3½ hours the levels of the amino acids in the blood of these patients either remained practically unaltered or tended to rise or fall by a few per cent from the initial postabsorptive level. The findings were in marked contrast to those observed after the administration of insulin or glucose. The controls also might be considered an added check on the methods used in this study.

Discussion. Our report in a previous publication[2] that there was a marked depression in the level of glutamine and total amino acids in the blood plasma of patients receiving insulin hypoglycemic shock treatment, was confirmed by Hamilton[18] in experiments on dogs. Since he found that the percentage drop of glutamine and of the total amino acids was the same he interpreted these findings as indicating that the drop in the level of glutamine was due merely to a diffusion of the amino acids from the blood into the tissues. We have suggested[19] that other interpretations of the data were possible and have discussed the possibilities in a previous publication.[2]

The findings in this study indicate that various amino acids in the blood differ in the extent to which they are affected during insulin hypoglycemic coma. Such differences are also found following the oral administration of glucose. It is apparent that some pronounced alterations in nitrogen metabolism, other than the uniform diffusion of amino acids from the blood into tissues, must occur which would account for the results obtained.

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[18] Hamilton, P. B., *J. Biol. Chem.*, 1945, **158**, 397.

[19] Harris, M. M., Roth, R. T., and Harris, R. S., *J. Nerv. and Ment. Dis.*, 1945, **102**, 466.

Bouckaert and his coworkers[20] and Mirsky[21] were of the opinion, from their studies on nephrectomized, hepatectomized, and eviscerated animals, that insulin inhibits deamination by the liver and also affects the synthesis and hydrolysis of proteins in the various tissues of the body. Since the injection of insulin into eviscerated animals resulted in the disappearance of endogenous amino acids from the blood stream, Mirsky[21] favored the view that this was due to an increase in synthesis rather than a decrease in hydrolysis of proteins in the peripheral tissues. Frame and Russell[22] have recently carried out similar studies on eviscerated rats and confirmed the findings. They point out that it is not known at present whether insulin promotes synthesis of tissue proteins from blood amino acids or inhibits its breakdown.

From the standpoint of the possible mechanisms involved it is of interest to analyze the changes observed in the level of the various amino acids in the blood as a result of insulin and glucose administration. It will be observed that, with the exception of arginine, and histidine, the amino acids thus far studied fall into the group of so-called essential amino acids. These amino acids may be synthesized in the body to only a very limited extent or not at all.[8] This should simplify, somewhat, the analysis of the changes observed. The figure for total amino acid nitrogen represents both essential and nonessential amino acids in the blood plasma.

It will be seen from Table I that in the controls the level of the amino acids shows little change during the 3½ hours of observation in the postabsorptive state. Following the administration of insulin or glucose (Tables I and II) there is a significant drop, but to a variable degree, in the levels of the various amino acids.

As the controls indicate, the patients, during the 3½ hours of observation, are approximately in a state of dynamic equilibrium as far as the level of the various amino acids in the blood are concerned. That is, the amino acids supplied to the blood as a result of protein hydrolysis or amino acid synthesis is about balanced by those removed from the blood for deamination and oxidation or protein or polypeptide synthesis.

Since insulin or glucose tends to spare protein metabolism one should expect a decrease in deamination and oxidation of amino acids. This is in keeping with the findings of Mirsky[21] and Bouckaert, and his co-workers[20] in their animal experiments. However, a decrease in deamination and oxidation of amino acids should tend to raise the level of amino acids in the blood. Since an appreciable drop in the level of amino acids is observed following glucose or insulin, an inhibition of hydrolysis of tissue proteins or an increase in the synthesis of polypeptides or proteins probably occurs to account for the findings.

If, in the various patients, insulin or glucose inhibited the hydrolysis of the same type of storage protein or increased the synthesis of some polypeptide or protein of similar composition, then the ratios of the amounts of the various amino acids disappearing from the blood stream should be similar in the different patients. This tendency occurs for some of the amino acids in some of the patients. However, there are many significant deviations. Thus, for example, patient (J.B., No. 1) in Table I shows a drop of 37 γ of leucine and 18 γ of lysine per ml of plasma. This is a ratio of leucine to lysine of approximately 2. In patient (A.J., No. 5), leucine dropped 41 γ and lysine only 10.5 γ which gives a ratio of leucine to lysine of approximately 4. Thus the mechanisms which depress the level of the amino acids, thrown into action as a result of the insulin hypoglycemia, may not be identical in the 2 patients. This marked difference in the handling of essential amino acids may be highly significant.

In the case of the nonessential amino acids, arginine and histidine, it will be seen that there are marked differences in the ratios of the amounts of these amino acids which disappear from the blood plasma. Thus, for example, patient (J.M., No. 2) showed a drop of 8.6 γ of arginine and 9.8 γ of histidine per ml of plasma while in patient (A.M., No. 6) 18.8 γ of arginine and 6.2 γ of histidine disappeared from the plasma. Thus, while more than twice the amount of arginine disappeared in the latter as compared with the former patient, as regards histidine the situation was reversed. Thus the extent of the relative effect of insulin hypoglycemia on arginine and histidine formation and utilization must be significantly different in these patients. The studies of previous investigators regarding the effect on the level of total amino acids in the blood failed to reveal the complexity of the metabolic processes which require consideration. General speculation as to hydrolysis and synthesis of tissue protein to account for the marked effects of insulin and glucose administration would appear to be a rather crude approach to complex metabolic processes.

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[20] Bouckaert, J. B., and de Duve, Chr., *Physiol. Rev.*, 1947, **27**, 39.

[21] Mirsky, I. A., *Am. J. Physiol.*, 1938, **124**, 569.

[22] Frame, E. G., and Russell, J. A., *Endocr.*, 1946, **39**, 420.

In some patients the oral administration of glucose produced as marked a depression in the level of valine and arginine as that seen in insulin hypoglycemic coma. (See patient J.F., No. 10, Table II). However, the effect on the other amino acids was not comparable. This points to some special effects of insulin aside from the supply and utilization of glucose on the metabolism of some of the amino acids. This seems to be especially marked as regards leucine and lysine of the amino acids thus far investigated. In this connection it may be of interest to point out that Steele and his co-workers[23] recently have found that leucine is one of a few amino acids which is not excreted in human urine, either free or combined, except under very special conditions. This occurs in spite of the comparatively high level of leucine in the blood. These findings may point to some special or unique function of leucine in the nitrogen metabolism of man. This will be investigated further.

The possible relation of the amino acids to clinical conditions will be the subject of another paper.

Summary. 1. The effect of insulin hypoglycemic coma and of the oral administration of glucose on the level of various amino acids in the blood have been investigated in 15 mental patients.

2. The level in the blood of all the amino acids, thus far investigated, decreases to a variable extent during insulin hypoglycemia and also after glucose administration.

3. Insulin hypoglycemia produced a more marked effect on the level of blood amino acids than did the administration of glucose. Valine appeared to be an exception in some patients.

4. Leucine and lysine showed the most marked changes of the amino acids thus far investigated.

5. The total amino acids does not reflect the extent of the changes in the blood of the level of the individual amino acids.

6. The possible mechanisms which may account for the changes are discussed.

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[23] Steele, B. F., Sauberlich, H. E., Reynolds, M. S., and Baumann, C. A., *J. Nutrition*, 1947, **33**, 209.

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Studies on Cigarette Smoke Irritation. I. Determination of Edema-Producing Properties of Certain Types of Cigarette Tobaccos.

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We described[1] a method for the quantitative evaluation of cigarette smoke irritation, as judged by its edema-producing properties, based on gravimetric determination of the increase in moisture content of the upper

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[1] Finnegan, J. K., Fordham, Doris, Larson, P. S., and Haag, H. B., *J. Pharm. and Exp. Therap.*, 1947, **89**, 115.

TABLE I.
Comparative Edema-Producing Properties of Certain Types of Cigarette Tobaccos.

Type of cigarette	No. of observations	Mean of ratio differences*	Mean of % increase in moisture
A. Smyrna (Turkish)	20	0.56	11.82
B. Bright	20	0.96	21.76
C. Maryland	20	1.38	30.76
D. Samsoun (Turkish)	20	1.47	33.79
E. Burley	20	1.71	37.12
F. Blend of A-E	50	1.47	31.14

* Ratios for exposed tissues minus ratios for control tissues.

TABLE II.
Significance of the Differences Observed Between the Edema-Producing Properties of the Types of Cigarette Tobaccos Listed in Table I.

Comparison	P value*	Comparison	P value	Comparison	P value
A vs B	.41	B vs C	.31	C vs E	.39
A vs C	.01	B vs D	.29	C vs F	.76
A vs D	.02	B vs E	.10	D vs E	.70
A vs E	.002	B vs F	.12	D vs F	.98
A vs F	.002	C vs D	.82	E vs F	.44

* Probability values of .05 and less are usually held to indicate statistical significance; .01 and less indicate a high degree of significance.

palpebral conjunctiva of the rabbit eye following its exposure to cigarette smoke. The method is briefly as follows:

Using a smoking machine of the type described by Bradford, Harlan and Hanmer[2] which draws a 35 ml puff once per minute, 3 puffs are directed through a suitable eye cup placed over the widely opened eye of a morphinized rabbit. When one eye only of each rabbit is exposed, the other serving as a control, we have named the technic "Procedure A." Where both eyes of each rabbit are exposed, thereby permitting comparison of 2 cigarettes on the same animal, the technic is called "Procedure B." In both procedures, following exposure to the smoke, a one-hour period is allowed for edema formation. The rabbit is then sacrificed, the upper lid membrane of each eye excised, weighed, dried in an oven to constant weight, and reweighed. From the wet and dry weights the ratio of moisture to dry weight is calculated and, in the case of Procedure A, the per cent increase in water in the exposed membrane over that in the control membrane is determined. In a series of such determinations involving 2 or more groups of cigarettes, the differences in the ratios between groups are evaluated statistically for significance by analysis of variance.

In the present study, we have applied Procedure A to an investigation of a blend of cigarette-tobacco types in an effort to evaluate the role of each in the production of cigarette smoke irritants of the edema-producing type.

Experimental. Six groups of cigarettes were prepared, one containing only Smyrna (Turkish) tobacco, one containing only Samsoun (Turkish) tobacco, one containing only Bright tobaccos, one containing only Maryland tobaccos, one containing only Burley tobaccos, and one containing a blend of all of these tobaccos. All were brought to comparable moisture content (11.5-12.5%) by placing them in atmospheres of suitable humidity. From each group, cigarettes were selected for smoking purposes on the basis of comparable resistance to air flow through them when subjected to a slight constant negative pressure at one end. Fifty of the blended cigarettes and 20 from each of the other groups were tested by Procedure A and the results are recorded in Table I and the significance of differences in Table II.

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[2] Bradford, J., Harlan, W., and Hanmer, H. R., *Ind. Eng. Chem.*, 1936, **28**, 836.

Discussion. From Tables I and II it is apparent that the smoke from certain types of cigarette tobaccos tends to be lower in edema-producing irritants than that from other types, and that in a blend of these tobaccos these irritants appear to be additive.

In our original description of the method here used[1] and throughout the presentation of the present study, we have stressed the fact that the only irritants measured by this technic are those that produce edema. That this is not necessarily related to the sensations of subjective irritation experienced by a smoker was strikingly brought out in the present study in the case of the cigarettes made from Burley tobaccos alone. As measured by edema-producing irritants alone this cigarette was not significantly more irritating than the blended cigarette, yet the sensations experienced by the smoker were quite the opposite. Whereas, the average smoker had no difficulty in making a deep inhalation of the smoke from the blended cigarette, a similar inhalation of smoke from the Burley cigarette left him unable to draw a second breath for an uncomfortably long period. It is apparent from this that substances which produce a degree of subjective irritation disproportionately greater than that which might be expected on the basis of their edema-producing properties, may be present in cigarette smoke. Development of a method capable of quantitative measurement of the subjective sensation of irritation to complement the measurement of edema-producing irritants so that a more complete picture may be obtained is greatly to be desired.

Conclusions. 1. Smoke from different types of cigarette tobaccos may differ significantly in edema-producing irritants. 2. Constituents are present in cigarette smoke, from at least certain types of tobaccos, which produce a degree of subjective irritation disproportionately greater than that which might be expected on the basis of their edema-producing properties.

We are indebted to Mrs. Sarah Guerry for technical assistance in performing the reported experiments.

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New Formula for Dilution Curve of Plasma Prothrombin. Normal Standards and Changes in Pathologic Conditions.

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The various one-stage methods for the measurement of prothrombin time used at present, differ little from the procedure originally employed.[1] They are based on the assumption that the clotting time of plasma is dependent solely on its prothrombin content, if an excess of thromboplastin and optimal amounts of calcium are provided; constancy of the rate of conversion of prothrombin to thrombin is assumed. As source of thromboplastin an extract of rabbit brain, or the venom of the Russel viper, are most commonly used.

In all procedures the quantitative comparison of abnormal with normal plasma meets with as yet unresolved difficulties, which have occasioned a variety of methods of assay. The simplest assumption,[2] that the clotting time of plasma is linearly proportional to its prothrombin content, is obviously incorrect,[3] since it can be shown that the clotting time of normal plasma on dilution follows a curve resembling a hyperbola in shape.[1,4] This curve shares with the hyperbola the property that the product of the 2 coordinates, clotting time in seconds and percentage of plasma concentration, tends to be constant but differs from a hyperbola in 2 respects: (1) a plasma percentage over 100 *a priori* cannot exist, and (2) the approach to infinity of clotting time in the region of low plasma concentrations is not truly asymptotic; beyond a certain dilution, plasma will no longer clot at all. In comparing abnormal plasma with the normal, generally the assumption has been made, either expressly or implicitly, that the clotting properties of abnormal plasma can be expressed in terms of a dilution of normal plasma. Often the prothrombin content has been expressed as per cent of normal, based on a curve established for serial dilutions of normal plasma. If the assumption underlying such a procedure were correct, dilution curves of normal and abnormal plasmas could be made to coincide by proper linear shifting of their curves along one of the coordinates. For instance, an abnormal plasma which undiluted might have a clotting time corresponding to a 50% concentration of normal plasma, on dilution with an equal volume of saline should give a clotting time corresponding to a 25% concentration of normal plasma. Serious doubts concerning the validity of this premise have been expressed, and it has been demonstrated that the dilution curves of pathic and normal plasmas usually cannot be superimposed, indicating that not only their starting points, but also their slopes differ.[5,6] One group of investigators, for purposes of assay of prothrombin-lowering substances, plotted the logarithm of the clotting time versus the logarithm of plasma concentration.[5] This method also is based on the assumption that the curves of normal and abnormal plasma have the same slope. No proof was adduced for this premise.

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[1] Quick, A. J., Stanley-Brown, M., and Bancroft, F. W., *Am. J. M. Sc.*, 1935, **190**, 501; Quick, A. J., *J. A. M. A.*, 1938, **110**, 1658.

[2] Ziffren, S. E., Owen, C. A., Hoffman, G. R., and Smith, H. P., *Am. J. Clin. Path., Tech. Supp.*, 1940, **4**, 13; Ziffren, S. E., Owen, C. A., Hoffman, G. R., and Smith, H. P., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 595; Govan, C. D., *J. Pediat.*, 1946, **29**, 629.

Some observers have preferred either a 12.5 or a 25% concentration of plasma for the routine measurement of prothrombin,[6,7] since in states of prothrombin deficiency the clotting time of plasma increases more on dilution than normally. One investigator,[8] noting considerable variation in the difference between the clotting times of undiluted plasma and a 1:8 plasma dilution in various clinical conditions, proposed that this difference was a more reliable index of the prothrombin activity than the clotting time of either undiluted or diluted plasma alone; when the difference between undiluted and diluted plasma was increased a state of hypoprothrombinemia was indicated, and either hyperprothrombinemia, or an increase of coagulation-inhibitors, when the difference was reduced.

Finally, a formula describing the dilution curve of normal plasma of rabbit and man has been given as follows:[9]

$$t = a + k/c$$

where t is the clotting time of any given concentration of plasma, c is the concentration of plasma, and a and k are constants. a is the intercept at infinite concentration, while at 100% concentration $t - a = k/100$. a was found to be 5.1 for rabbit and 10.3 for human plasma, while the k values were 139 and 354, respectively. This formula, indicating direct proportionality between the clot-

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[3] Quick, A. J., *The Hemorrhagic Diseases and the Physiology of Hemostasis*, Springfield, Ill., 1942, C. C. Thomas.

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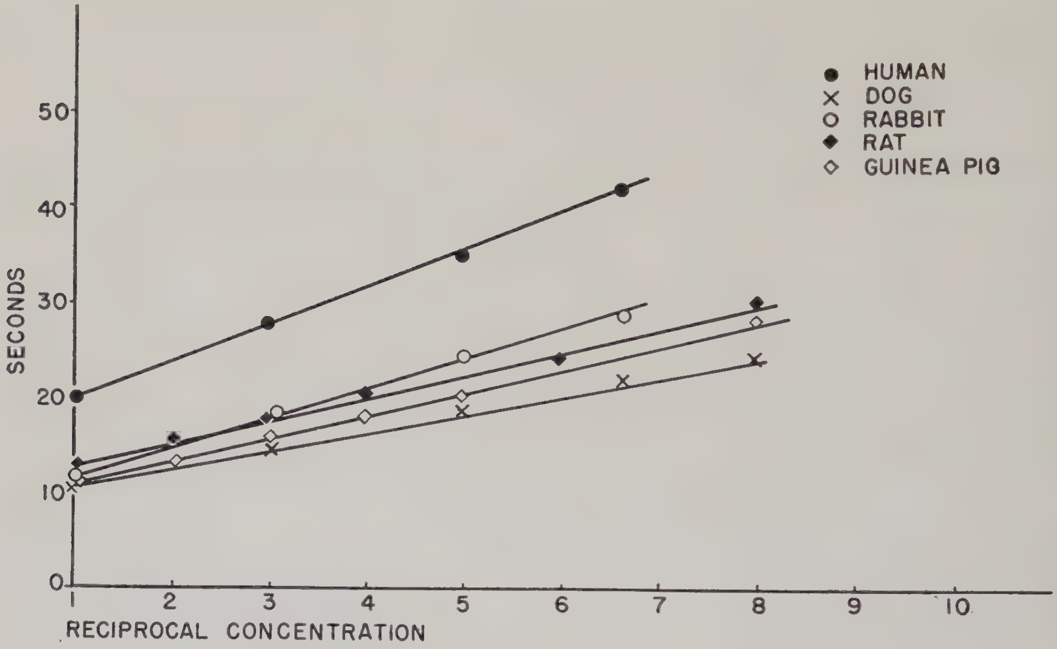

Fig. 1.
Prothrombin time and dilution constant of the blood plasma of man, dog, rabbit, rat and guinea pig.

ting time and $1/c$, the reciprocal of the concentration, fits the experimental finding that for any given plasma the product of clotting time and dilution tends to be constant. The authors did not use the formula for the description of abnormal plasma.

In the following it will be shown that the prothrombin time of normal as well as abnormal plasma of man, dog, rabbit, rat and guinea pig may be expressed by the following formula:

$$t = t_1 + k/(\text{dilution}^* - 1)$$

where t is the clotting time in seconds of any given plasma dilution,* t_1 that of undiluted plasma, and k is a proportionality factor, the dilution constant, which indicates the increase of clotting time per unit increment of reciprocal concentration. This formula, the equation of a straight line, indicates as do some formulas previously mentioned, direct proportionality between the reciprocal of plasma dilution and the clotting time but differs from them in the choice of the constants. Both t_1 and k are constants for any given plasma, but vary among different samples and under pathologic conditions. Considered analytically the 2 constants, representing the intercept and the slope of the line, are sufficient to describe the values of serial dilutions of any given plasma. In this communication are presented the normal standard values of t_1 and k for human, dog and rabbit plasma, 2 examples of the behavior of the constants in experimentally-produced hypoprothrombinemia, and a survey of their variations in clinical conditions.

Experimental. Freshly drawn blood, collected by venipuncture with precautions to avoid hemolysis and foaming, was delivered into test tubes measuring 10 x 75 mm and graduated at 1 cc, which contained 0.1 cc of 0.1 molar sodium oxalate. After gentle but thorough mixing the tubes were centrifuged for 5 minutes at 2000 r.p.m. The supernatant plasma was drawn off and the prothrombin time was determined according to the slightly modified method of Fullerton[10] as follows: an 0.15 cc aliquot of the oxalated

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* Dilution = the ratio of the final volume of the plasma-saline mixture to the original volume of plasma.

[10] Fullerton, H. W., *Lancet*, 1940, **2**, 195.

TABLE I.
Mean Normal Values for the Prothrombin Time of Undiluted Plasma (t_1) and the Dilution Constant of Man, Dog, and Rabbit.

All values are given in seconds.

Species	No. of samples	Undiluted plasma Mean	S.D.*	S.E.*	Dilution constant Mean	S.D.*	S.E.*
Man	37	19.9	1.2	0.2	3.8	0.9	0.2
Dog	22	11.4	1.4	0.3	1.8	0.5	0.2
Rabbit	10	11.4	1.4	0.4	3.2	0.5	0.4

* S.D. = standard deviation; S.E. = standard error.

plasma was measured into a small test tube and placed for one minute in an all-glass water bath permitting close observation at 37°. Then an equal amount of a similarly prewarmed mixture of thromboplastin and calcium chloride was added, and with constant shaking of the tube in the water bath the time for the formation of a solid fibrin clot recorded. Clotting times were also measured on various dilutions of plasma prepared with 0.9% sodium chloride solution. All determinations were performed in duplicate. The mixture of thromboplastin with calcium chloride was prepared by dissolving 0.5 mg of Russel-viper venom (Stypven, Burroughs-Wellcome Laboratories, 9 East 41st Street, New York, N. Y.) in 10 cc of 0.025 molar calcium chloride solution. The mixture was found stable for at least one month when kept in the refrigerator between tests. No significant differences between different batches of venom were ever noted over a period of 4 years, except in 1945-46 when owing to war-time shortages difficulties were experienced by the manufacturer in the preparation of a satisfactory product.

Results. 1. Normal values. In Fig. 1 are presented representative data on individual samples of normal human, dog, rabbit, rat and guinea pig plasma. As can be seen, a straight line relationship obtains for the range of dilutions studied. Fig. 1 also illustrates the fact that there is little relation between the clotting time of undiluted plasma and the dilution constant among different species. For instance it is apparent that the clotting times of the undiluted plasma of dog and rabbit are similar while the slopes of the dilution curves differ widely. On the other hand, rabbit and human plasma give similar dilution constants, despite the difference in the clotting time of the undiluted plasmas.

In Table I are given the mean values of the clotting time of undiluted plasma and the dilution constant, for human, dog and rabbit plasma together with their statistical description. For human plasma, if one accepts the conventional limits of ± 2 standard deviations as the limits of normal variation, a range of 17.5-22.2 seconds for the undiluted plasma and 2.0-5.6 for the dilution constant may be given. A proportional range is found for the plasmas of dog and rabbit.

The formula appears valid over a dilution range up to approximately 1:10 for normal

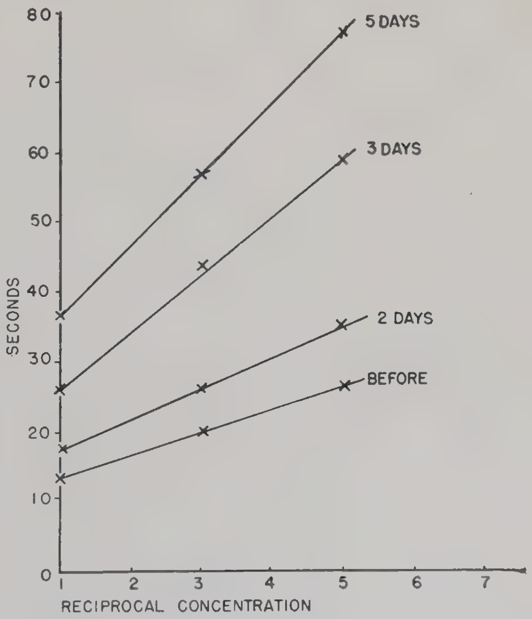

Fig. 2.
The prothrombin time of a rabbit following the subcutaneous injection of 0.5 cc per kg of methyl salicylate.

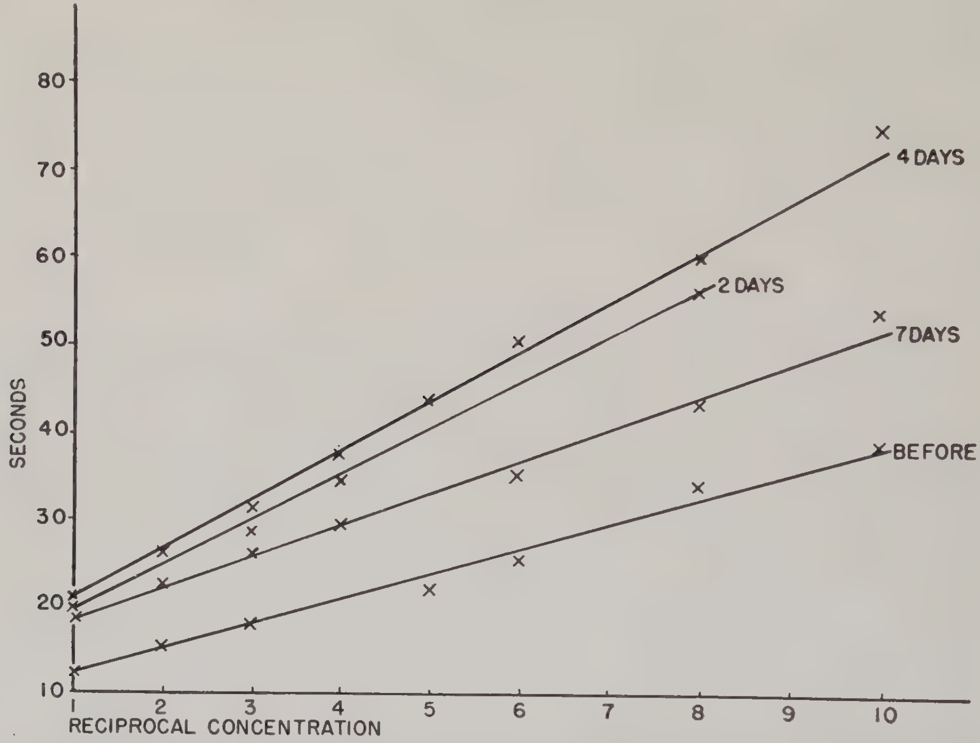

Fig. 3.
Changes of the prothrombin time of a rabbit following the intravenous injection of 2 mg per kg of dicumarol.

human and approximately 1:20 for dog plasma. On further dilution disproportionate lengthening of the clotting time occurs. In plasma with prolonged clotting time the proportionality range is reduced, suggesting the existence of an upper limit of approximately 70-80 seconds for the validity of the formula.

2. Changes in experimentally-produced hypoprothrombinemia. Two examples of hypoprothrombinemia produced in rabbits by the administration of dicumarol and methylsalicylate are presented in Figs. 2 and 3. It may be seen that in both experiments the undiluted plasma clotting time as well as the dilution constant increased. Such data indicate that both indices are related to the prothrombin content of the plasma, and that the curves of abnormal and normal plasma cannot be superimposed.

3. Changes in clinical conditions. The purpose of this section is not so much to present a compilation of changes of prothrombin time in disease, as to indicate the relation of the clotting time of undiluted plasma to the dilution constant. The patients for the most part were children. The data are divided into 2 groups on the basis of the dilution constant. In Table II are presented in order of increasing value cases of elevation of the dilution constant. They are recruited mostly from 3 groups: (1) cases of deficiency of vitamin K, (2) liver damage of various kinds, and (3) bleeding for extended periods of time. A fourth group of cases, patients on salicylate medication, is separately presented in Table IV.

It may be seen that there are interesting parallelisms as well as discrepancies in the behavior of the clotting time of undiluted plasma (t_1) and the dilution constant. In all cases of vitamin K deficiency, with marked prolongation of t_1 the dilution constant also was elevated. On the other hand in patients with severe hepatic damage the dilution constant was increased independently of the changes of undiluted plasma, an observation which has proved to be of diagnostic and prognostic value. The discrepancy between

TABLE II.
Cases of Prolonged Dilution Constant. (Listed in order of increasing value).

Case No.	Plasma prothrombin time Undiluted plasma (t_1) seconds	Dilution constant seconds	Diagnosis
1.	45.5	6.0	Aplastic anemia, bleeding profusely
2.	30.9	6.1	Chronic diarrhea
3.	25.7	7.2	Syphilitic hepatitis
4.	18.4	7.3	Congenital atresia of the bile ducts
5.	36.3	7.3	Lymphosarcoma, bleeding
6.	29.5	7.3	Histoplasmosis
7.	36.1	7.5	Infectious hepatitis, convalescing
8.	28.5	7.6	” ” ”
9.	33.9	7.6	Chronic diarrhea
10.	35.8	7.7	Intrahepatic biliary obstruction
11.	21.0	8.2	Cirrhosis of liver
12.	26.6	8.6	Rheumatic fever, cardiac decomposition with hepatic congestion
13.	26.7	8.7	Thrombocytopenic purpura
14.	15.0	8.8	Erythroblastosis fetalis
15.	27.2	9.0	Banti's disease
16.	18.4	9.3	Hemophilia, prolonged bleeding from gastro-enteric tract
17.	28.1	9.9	Rheumatic fever with congestion of liver
18.	28.7	9.9	Erythroblastosis fetalis, severe
19.	24.6	10.0	Typhoid fever, severe, 1 day premortem
20.	30.9	10.0	Hemophilia, epistaxis of 5 days' duration
21.	43.0	10.1	Erythroblastosis fetalis, severe anemia
22.	31.2	10.4	Banti's disease
23.	30.1	10.5	Congenital atresia of bile ducts, bleeding
24.	19.6	10.8	Cirrhosis of liver
25.	15.1	10.9	” ” ”
26.	34.0	10.9	Chronic diarrhea
27.	25.1	11.2	Infectious hepatitis of long duration
28.	23.2	12.4	Diarrhea
29.	21.5	13.8	Hepato-renal syndrome of lupus erythematosus
30.	32.3	14.3	Letterer-Siwe's disease, 1 day pre-mortem
31.	51.2	14.4	Acute infectious hepatitis with subacute liver atrophy
	27.9	15.5	1 day after vitamin K, 1 day pre-mortem
32.	20.0	14.9	Cirrhosis of liver
33.	29.4	16.4	Erythroblastosis fetalis, severe, 8 hours pre-mortem
34.	21.6	19.1	Erythroblastosis fetalis
35.	52.6	$\cong$32	Chronic diarrhea
36.	61.8	$\cong$37	” ”
37.	66.8	$\cong$44	Hemorrhagic disease of the newborn
38.	80.2	$\cong$56	Chronic diarrhea
39.	76.8	$\cong$75	” ”

the 2 indices in cases of hepatic damage as compared with their parallelism in vitamin K deficiency, suggests that humoral clotting factors other than prothrombin may play a role in hepatic disorders. The data on cases of prolonged bleeding, here presented, indicate that the immediately available supply of prothrombin, or vitamin K, may be exhausted. Thus patients with hemophilia may also develop hypoprothrombinemia (see cases 16 and 20). The data on the effect of salicylate medication in the usual dosage (0.1-0.15 g per kg body weight) on patients with rheumatic fever suggest that with parallel changes of the undiluted plasma and the dilution constant, the latter is a more sensitive indicator of the changes of prothrombin.

The cases of decrease of the dilution constant are drawn from a more heterogeneous material than those of prolongation. In Table III 3 groups appear prominent: (1) cases of infectious hepatitis in an early stage, (2) cases of the nephrotic syndrome, and (3) cases of rheumatic fever. Also represented are cases of purpura, leukemia, and Rocky Mountain Spotted Fever, disorders with bleeding

TABLE III.
Cases of Reduction of the Dilution Constant. (Listed in order of increasing value.)

	Plasma prothrombin time		
Case No.	Undiluted plasma (t_1) seconds	Dilution constant seconds	Diagnosis
40.	17.7	—0.4	Post-infectious state, with high serum agglutinin titer
41.	21.0	0.0	Non-thrombocytopenic purpura
42.	22.5	0.4	Nephrotic syndrome
43.	30.0	0.4	,, ,,
44.	16.0	0.5	Infectious hepatitis
45.	23.8	0.5	Symptomatic purpura
46.	19.9	0.6	Nephrotic syndrome
47.	17.1	0.7	Post-tonsillectomy bleeding
48.	21.7	0.8	Infectious hepatitis
49.	30.6	0.8	Acute lymphatic leukemia
50.	16.4	1.3	Infectious hepatitis
51.	20.7	1.4	Nephrotic syndrome
52.	15.2	1.5	Rheumatic fever
53.	29.0	1.5	Rheumatoid arthritis
54.	18.3	1.7	Nephrotic syndrome
55.	22.5	1.7	Rocky Mountain spotted fever
56.	14.8	1.8	Acute lymphatic leukemia
57.	16.6	1.8	Rocky Mountain spotted fever
58.	21.7	1.8	Infectious hepatitis
59.	14.7	2.0	Sickle cell anemia in hemolytic crisis
60.	19.9	2.0	Rocky Mountain spotted fever
61.	20.8	2.0	Nephrotic syndrome
62.	14.4	2.1	Rheumatic fever
63.	16.1	2.1	,, ,,
64.	16.4	2.1	Infectious hepatitis
65.	22.3	2.1	Rheumatic fever

from the small vessels as a common feature. Another group, cases of patients receiving vitamin K, is presented in Table V.

Again, a widely varying relationship between t_1 and the dilution constant is evident. While both indices changed in a parallel manner in some patients with infectious hepatitis and in those receiving vitamin K, marked discrepancies were found among the other patients. Of particular interest is the combination of a considerable prolongation of t_1 with a marked reduction of the dilution constant (see cases 43, 49 and 53). Whether the reduced dilution constant reflects an actual increase of prothrombin content or is the result of an increase in the amount of auxiliary clotting factors cannot be said at the present. Reduction of both t_1 and the dilution constant suggests strongly the existence of a state of true hyperprothrombinemia but additional effects of activators and inhibitors of clotting have to be invoked in cases where the 2 indices differ from each other.

Discussion. It may be well to point out that no definite independent biological meaning has been assigned to the 2 constants in the expression for prothrombin time. The data on hypoprothrombinemia, experimental as well as clinical, suggest that the constants, not truly independent of each other, change in a parallel manner. On the other hand, the observations on different species and on the clinical material illustrate marked and well defined discrepancies between the 2 indices. These differences may be related to variations in the amount of auxiliary factors of clotting rather than of prothrombin.

Clinically of greatest interest is the apparent usefulness of the dilution constant as an indicator of hepatic damage. In infectious hepatitis decreases as well as increases of the dilution constant occur, suggesting the occurrence of a period of stimulation, followed by suppression, of hepatic function. Similar changes in cases of bleeding may be due to stimulation followed by exhaustion of prothrombin formation. Most of the conditions in which a decrease of the dilution constant was observed are characterized by altera-

TABLE IV.
Effect of Salicylate on Dilution Curve of Plasma Prothrombin.

Case No.	Plasma prothrombin time		Comments
	Undiluted plasma (t_1) seconds	Dilution constant seconds	
66.	20.1	4.9	Before
	26.6	9.1	2 days salicylate
	25.5	9.7	4 ’’ ’’
	26.8	10.6	6 ’’ ’’
	25.8	11.5	8 ’’ ’’
	23.2	9.6	2 ’’ stopped
67.	20.2	5.6	Before
	24.7	6.4	2 days salicylate
	22.9	6.8	4 ’’ ’’
	22.1	5.7	2 ’’ stopped
68.	16.2	2.2	Before
	24.1	5.0	4 days salicylate
	27.5	6.8	7 ’’ ’’
	19.6	4.7	5 ’’ after
69.	21.0	3.7	Before
	23.9	5.8	3 days salicylate
	23.9	6.7	6 ’’ ’’
	20.2	4.7	4 ’’ after
70.	20.8	5.2	Before
	31.1	10.3	4 days salicylate
71.	22.0	4.7	Before
	22.9	7.1	4 days salicylate
	20.5	5.5	2 ’’ after
72.	19.1	4.7	Before
	21.3	6.0	2 days salicylate
	20.3	6.9	6 ’’ ’’
73.	19.7	3.5	Before
	21.4	6.4	2 days salicylate
	22.6	7.2	7 ’’ ’’
74.	20.2	2.4	Before
	27.6	9.6	12 days salicylate
	22.0	2.0	10 ’’ after

TABLE V.
The Effect of Vitamin K Medication on Dilution Constant.

Case No.	Plasma prothrombin time		Comments
	Undiluted plasma (t_1) seconds	Dilution constant seconds	
10.	35.8	7.7	Intrahepatic biliary obstruction
	16.1	0.9	2 days after Vit. K
37.	66.8	≅44	Hemorrhagic disease of new born
	16.2	1.4	1 day after Vit. K
39.	76.8	≅75	Chronic diarrhea
	14.7	1.7	2 days after Vit. K
27.	25.1	11.2	Infectious hepatitis of long duration
	19.2	2.0	2 days after Vit. K
75.	15.4	1.4	Erythroblastosis fetalis 1 day after Vit. K
76.	16.3	1.8	Chronic diarrhea 1 day after Vit. K

tions in the distribution of plasma protein and increases of fibrinogen. Such patients may experience a general humoral stimulation in which clotting factors participate.

Summary. A new formula expressing the dilution curve of plasma prothrombin is presented. Normal standards and examples of change in experimental clinical conditions are given.

15837

Inhibitory Effect of Dibenamine on Vasoconstrictor Substances.

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Recently Nickerson and Goodman[1] reported the results of the study of a new adrenolytic and sympathicolytic drug, dibenzyl-β-chloroethyl amine hydrochloride (dibenamine). They found that after the administration of this agent to dogs and cats, intravenous epinephrine in high doses causes a reversal of blood pressure (a fall of 20 to 40 mm Hg). In addition to the blocking and reversing of the excitatory adrenergic response, dibenamine had the ability to prevent epinephrine-induced cardiac irregularities in dogs sensitized by cyclopropane.[2] Most of the studies on dibenamine reported above deal with responses of heart rate, blood pressure, nictitating membrane, pupillary reactions.

Because of the possible clinical usefulness of dibenamine in angiospastic conditions associated with peripheral vascular diseases, causalgic states or hypertension,[†] an investigation of vasomotor responses to this agent, as expressed in terms of peripheral blood flow, seemed of interest. The present report deals with a study of the inhibitory effect of dibenamine on vasoconstrictor substances.

Methods. All the experiments reported in this investigation were carried out in the Laewen-Trendelenburg (L-T) preparation in frogs *(Rana pipiens)*. This preparation is a sensitive indicator in testing vascular responses, and offers a simple tool for the analysis of vasomotor agents and their antagonists. The perfusate used was a solution containing 0.65% NaCl and 0.02% KCl. Two Mariotte bottles were connected by a Y-piece with the perfusion system, *i.e.* the rubber tubing leading to the aortic cannula. One bottle contained the plain perfusion solution, the other, added to the latter, dibenamine.[‡] This substance was used at various concentrations. Epinephrine hydrochloride, nicotine base (Eastman Kodak) and hypertensin (angiotonin)[§] were used in testing the vasoconstrictor-blocking ability of dibenamine. The injections of these vasopressor substances were made through the rubber tubing into the glass cannula inserted into the abdominal aorta of the frog. Their vasomotor activity was evaluated in terms of the output variations of drops per minute.

Results. The injection of a small amount of epinephrine (1/10-1 γ), nicotine (1-10 γ) or hypertensin (0.05-0.5 cc) in the L-T preparation induces a marked vasoconstriction as

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[*] Elsa and William Menke Fellow.

This investigation was aided by a grant from the Peripheral Vascular Disease Research Fund.

[1] Nickerson, M., and Goodman, L. S., *Fed. Proc.*, 1946, **5**, 194.

[2] Nickerson, M., Smith, S. M., and Goodman, L. S., *Fed. Proc.*, 1946, **5**, 195.

[†] Clinical investigation of the action of dibenamine in such conditions is now in progress.

[‡] Dibenamine was supplied by Givaudan-Delawanna, Inc., Delawanna, N.J., through the courtesy of Dr. W. Gump.

[§] Hypertensin (angiotonin) was prepared from the interaction of Renin (Smaco) and sheep serum according to the method described by Sapirstein *et al.*[3]

[3] Sapirstein, L. A., Reed, R. K., and Southard, F. D., Jr., *J. Lab. and Clin. Med.*, 1944, **29**, 633.

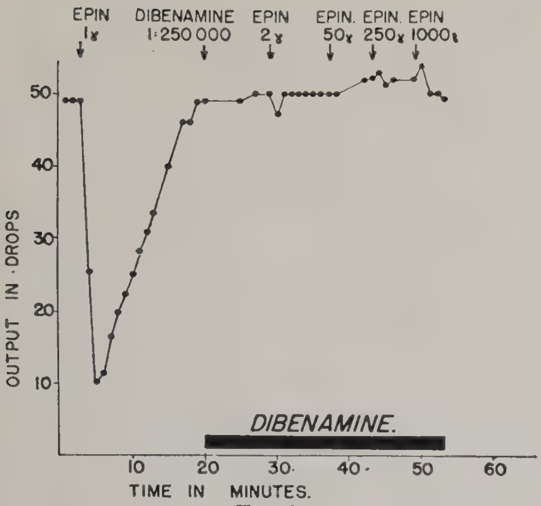

FIG. 1.
Inhibitory effect of dibenamine on the vasoconstrictor action of epinephrine. (The bar indicates the time of perfusion with dibenamine).

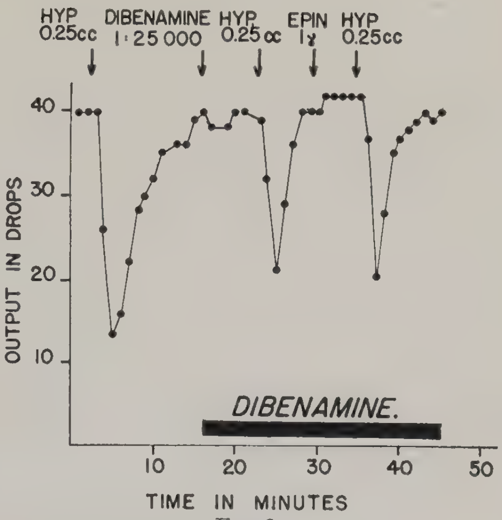

FIG. 2.
Effect of dibenamine on hypertensin. (The bar indicates the time of perfusion with dibenamine).

expressed by the reduction of the output (50-80%) and the duration of this effect ranging from 10 to 30 minutes or more, *i.e.* before the output returns to its initial level. Each individual preparation was first tested for its sensitivity to epinephrine as well as to the other pressor substances used in this investigation.

I. *Effect of dibenamine on epinephrine.* (a) Dibenamine hydrochloride in a concentration of 1:2,500,000 or less inhibits the action of epinephrine only partially. In higher concentrations ranging from 1:1,000,000 to 1:250,000 or more, dibenamine inhibits the vasoconstrictor action of epinephrine completely within 5 to 10 minutes after the onset of the perfusion. The outstanding feature of this effect, as the perfusion with dibenamine progresses (20 to 30 minutes), is a complete inhibition of the response to even large amounts of epinephrine (50-1000 γ) (Fig. 1).

(b) Dibenamine hydrochloride was kept in solution together with epinephrine (100 γ of epinephrine in 50 cc of a solution of 1:250,000 dibenamine) *in vitro* for 30 to 60 minutes. When such epinephrine was tested after that lapse of time, its vasoconstrictor activity was essentially unaltered.

(c) According to the chemical constituents present in the perfusate, dibenamine exhibited a more or less complete inhibitory action on large amounts of epinephrine. The perfusing solution containing 0.65% NaCl and 0.02% KCl is the most suitable vehicle, if dibenamine is to show a complete blockade of large amounts (50-1000 γ) of epinephrine. The bicarbonate-free Tyrode solution is less suitable, for dibenamine does not then inhibit the vasoconstrictor action of large amounts of epinephrine completely. This seems to be due chiefly to the presence of calcium chloride. Indeed, when the perfusate contained 0.035% $CaCl_2$ in addition to 0.65% NaCl and 0.02% KCl, the adrenolytic action of dibenamine was greatly diminished for the large amounts of epinephrine.

II. *Effect of dibenamine on hypertensin (angiotonin).* Amounts of hypertensin ranging from 0.05 cc to 0.5 cc induce a marked and sustained constriction of the vascular bed of the frog's hind legs. 10 to 20 minutes after the onset of the perfusion with dibenamine, in concentrations ranging from 1:250,000 to 1:25,000, the vasoconstrictor action of hypertensin remains unchanged. After 40 to 50 minutes of perfusion its action is altered only slightly (Fig. 2).

III. *Effect of dibenamine on nicotine.* The injection of small amounts of nicotine (1-10 γ) in the L-T preparation elicits 2 major reactions: (a) twitchings of the thigh and leg muscles, (b) marked vasoconstriction.[4] Within 30 to 45 minutes after the onset of the perfusion with dibenamine (1:250,000 to 1:25,000) when the action of large amounts (50-1000 γ) of epinephrine is completely blocked, nicotine still induces notable vasoconstriction and visible twitchings of the muscles.

Discussion. Dibenamine hydrochloride used in concentrations of 1:1,000,000 to 1:25,000 prevents epinephrine-induced vasoconstriction as tested in the Laewen-Trendelenburg preparation. The protective effect afforded by this drug appears to be very potent, for it can block the action of very large amounts of epinephrine (1000 γ). This is practically a total adrenolytic effect. A comparative study with benzyl-imidazoline hydrochloride (priscol),‖ another synthetic autonomic blocking agent,[5] used under the same experimental conditions, exhibits a similar protective action but only for smaller amounts of epinephrine. This is in agreement with the results obtained by other investigators experimenting in intact animals.[2,6] The inhibitory effect of dibenamine on epinephrine does not seem to be due to a direct chemical interaction between the 2 molecules. The *in vitro* experiments reported above seem to support this view. In addition to being adrenolytic, dibenamine exhibits also a marked sympathicolytic action as shown by studies in the intact animal.[1,2] Since dibenamine is an adrenolytic and sympathicolytic agent, its site of action is probably at the neuroeffector cells or between the postganglionic nerve endings and the latter.

Within 30 to 50 minutes after the onset of the perfusion with dibenamine, when the action of large amounts of epinephrine is completely inhibited, small amounts of both hypertensin and nicotine still exhibit a marked vasoconstrictor activity. These facts seem to indicate that dibenamine is either specifically adrenolytic or that these various vasoconstrictor substances do not have the same site of action.

Summary. 1. Dibenzyl - β - chloroethyl amine hydrochloride (dibenamine) inhibits the vasoconstrictor action of epinephrine, as tested in the Laewen-Trendelenburg preparation in the frog. Its adrenolytic action is very potent, for it blocks even large amounts of epinephrine (50-1000 γ). 2. When the action of large amounts of epinephrine is completely inhibited by dibenamine, both hypertensin and nicotine still exhibit a marked vasoconstrictor activity. 3. The peripheral locus of action of dibenamine seems to be at the neuroeffector cells or between the postganglionic nerve endings and the latter.

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[4] Haimovici, H., and Pick, E. P., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 234.

‖ Priscol was supplied by Ciba Pharm. Prod., Summit, N. J., through the courtesy of Dr. E. Oppenheimer.

[5] Schnetz, H., and Fluch, M., *Z. f. Klin. Med.*, 1940, **137**, 667.

[6] Raab, W., and Humphreys, R. J., *J. Pharm. and Exp. Therap.*, 1946, **88**, 268.

15838

Effect of Thiouracil and Estrogen on Lactogenic Hormone and Weight of Pituitaries of Rats.*

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The weight of the hypophysis increases subsequent to thyroidectomy,[1-4] and this may be due to an augmentation of the basophil cells[4,5] which are presumed to secrete thyrotropin.[6–8] The acidophils, on the other hand, become markedly decreased following thyroidectomy.[9] The administration of goitrogenic substances, like thyroidectomy, can produce similar effects on the hypophysis. Thus, they have been reported to increase the weight of the pituitary,[10] increase and hypertrophy the basophil cells, and reduce the number and size of the eosinophils.[11–13]

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* Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series No. 1042.

[1] Hammett, F. S., *Am. J. Anat.*, 1923, **32**, 37.

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The acidophils of the hypophysis are the source of the lactogenic hormone,[6,7,14] and hence it would be expected that this hormone would be reduced following thyroidectomy. However, the only 2 reports available on this subject are not in agreement. McQueen-Williams reported that thyroparathyroidectomy in male rats reduced the lactogen content of the pituitary,[15] whereas Reineke, Bergman and Turner found no apparent change in the pituitary content of this hormone following thyroidectomy in young goats.[16]

It was thought that since thiouracil is similar in its effects to thyroidectomy, it would offer a considerable advantage in a restudy of the relation of thyroid deficiency to the secretion of hypophyseal lactogenic hormone. In the rat it would make unnecessary the surgical removal of the thyroids, with the inevitable concomitant removal of the parathyroids which creates an additional deficiency in the animal. It was also considered of interest to determine the effects of estrogen in combination with thiouracil on the lactogen content of the pituitary. Estrogen has been shown to be a powerful stimulator of lactogenic secretion, greatly increasing its content in the pituitary,[17–19] in the blood[20] and initiating milk secretion in several species.[18,21,22] Would thiouracil inhibit the dynamic effect of estrogen on pituitary lactogenic secretion?

Experimental. The first experiment ran for 24 days, and was performed on young female albino rats of a standard strain we have been using for years.† The control rats (Group 1) received no special treatment while Group 2 was fed a ration containing 0.1% thiouracil‡ during the 24-day period. Group 3 was given 100 I.U. estrone‡ daily during the last 10 days of the 24-day period, while Group 4 received 0.1% thiouracil for the first 14 days, and thiouracil plus 100 I.U. estrone daily during the last 10 days of the experiment.

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† We are indebted to Dr. A. G. Hogan, Chairman of the Department of Agricultural Chemistry, University of Missouri, for a generous supply of these rats.

‡ We wish to thank Dr. Mark Welsh of the Lederle Lab., Pearl River, N.Y., for the thiouracil; Dr. D. W. MacCorquodale of the Abbott Research Labs., North Chicago, Ill., for the estrone; and Dr. D. F. Green of Merck and Co., Rahway, N.J., for the diethylstilbestrol used in this study.

TABLE I.

Group	No.	Sex	Treatment	Avg body wt		Avg No. lactogen units*			Avg pit. wt, mg	Avg pit. wt†/ 100 g B.W.	Avg thyroid wt, mg	Avg thyroid† wt/ 100 g B.W., mg
				1st day	24th day	per pit.	per 100 g body wt	per mg pit. tissue				
				Experiment 1. Normal Female Rats.								
1	10	F	[1]Controls	108	130	8.5	6.0	1.25	6.76	5.18 ± .22	12.5	9.6 ± .61
2	9	F	[1]0.1% Thiouracil	109	129	5.5	4.2	0.61	8.93	6.90 ± .22	57.6	44.5 ± 2.85
3	10	F	[2]100 I.U. estrone daily from 14th	110	128	12.0	9.3	1.49	8.05	6.29 ± .17		
4	10	F	[2]0.1% Thiouracil + 100 I.U. estrone, 14th through 24th days	116	126	5.5	4.3	0.60	9.04	7.15 ± .20	46.0	36.5 ± 3.51
				Experiment 2. Castrate Male Rats.								
				1st day	21st day							
1	7	M	Controls	132	180	1.7	0.9	.18	9.17	5.09‡	10.6	6.5 ± .03
2	8	M	[3]10 γ Diethylstilbestrol daily	129	130	3.2	2.5	.41	7.81	6.02	12.8	9.5 ± .95
3	6	M	[3]10 γ Diethylstilbestrol + 0.1% Thiouracil daily	128	134	3.0	2.2	.25	11.60	8.69	53.1	38.8 ± 3.18

[1,2,3] Groups assayed and compared with each other in same 10 pigeons.

* Reece-Turner units.[17]

† Standard error of the mean.

‡ Individual weights were not available for determining S.E.M.

The second experiment§ ran for 21 days and included 3 groups of young male rats which had been castrated about 10 days earlier. Group 1 served as controls and received subcutaneous injections of .05 cc of corn oil daily. Group 2 was given injections of 10 μg of diethylstilbestrol‡ in corn oil daily while Group 3 similarly received 10 μg of diethylstilbestrol by injection plus 0.1% thiouracil in the feed daily.

All the rats were killed by etherization, and the pituitaries and thyroids were removed and weighed to the nearest .01 mg. Each group of pituitaries was macerated with a small mortar and pestle, suspended in an exact volume of distilled water, and the equivalent of 2 female or 6 male pituitaries was injected intradermally over one side of the crop glands of 10 pigeons. Two different groups of pituitaries were assayed and compared with each other in the same 10 pigeons by injecting over each side of the crop gland. The crop glands were rated for lactogen units by the Reece-Turner method.[17]

Results. Experiment 1. The lactogenic hormone in the pituitaries of the thiouracil-treated rats (Group 2) was reduced below that in the controls (Group 1), averaging 4.2 units compared to 6.0 units per 100 g body weight. The rats which received estrone alone (Group 3) showed a considerable increase in pituitary lactogen over the controls, averaging 9.3 units per 100 g body weight. Group 4, which received the same amount of estrone but got thiouracil in addition, not only showed no increase in lactogenic hormone over the control rats but had as little (4.3 units per 100 g body weight) as in the animals treated with thiouracil alone.

The pituitaries of the thiouracil, and thiouracil plus estrone-treated groups were definitely heavier than in the control rats, both increasing about the same amount per 100 g of body weight. The thyroids of both these groups weighed much more than in the controls, but the increase in thyroid weight was less in the group which received thiouracil plus estrone.

Experiment 2. It will be seen that in this experiment the lactogen content of the pituitaries of the thiouracil plus stilbestrol-treated rats (Group 3) rose just as much above the controls (Group 1) as the animals which received stilbestrol alone (Group 2). It must be pointed out that the Group 3 rats in this experiment do not correspond to the thiouracil plus estrone-treated group in the first experiment. The former received thiouracil and estrogen together during the entire 21-day period, while the latter got only thiouracil for the first 14 days and thiouracil with estrogen during the last 10 days of the 24-day period.

The pituitaries of the rats which were given thiouracil plus stilbestrol (Group 3) weighed considerably more than the animals treated with stilbestrol alone (Group 2) or the controls (Group 1). The average thyroid weight of the rats in Group 3 was increased approximately 5-fold over the controls.

Discussion. The reduction in the lactogen content of the pituitary following thiouracil treatment confirms the results reported by McQueen-Williams in thyroparathyroidectomized rats. This is believed to support the contention that the eosinophils, which are decreased following thyroidectomy or goitrogen treatment, are the source of lactogenic hormone. It is interesting that whereas the combination of thiouracil and estrogen in the first experiment did not evoke an increase in pituitary lactogen, it did do so in the second experiment. It is believed this difference can be accounted for in the somewhat different procedure followed with the 2 groups. Thus, the former received the estrogen only after a preliminary treatment with thiouracil for 2 weeks whereas the latter group received thiouracil and estrogen together during the entire length of the experiment. It would seem that in the first experiment the administration of thiouracil alone during a 2-week preliminary period reduced the eosinophils in the hypophysis to such a low level that they were rendered incapable of responding to subsequent estrogen stimulation and hence evoked no lactogen increase. In the second experiment on the other hand, the simultaneous administration of estrogen with thiouracil checked the depressing effect of the latter on the eosinophils and enabled the estrogen to evoke its normal increase of pituitary lactogen.

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§ This was part of another experiment by V. Hurst, J. J. Trentin and the authors to determine the effects of thiouracil plus estrogen on mammary growth.

The increase in the pituitary weight following thiouracil treatment corroborates a similar report on thiourea-treated rats,[10] and is in line with the findings in thyroidectomized animals. The recognized ability of estrogen to increase pituitary size was evident both in the estrone and diethylstilbestrol-injected rats. However, the greatest augmentation of pituitary weight occurred in the rats which received the above estrogens in combination with thiouracil.

Summary. The administration of 0.1% thiouracil in the feed for 24 days to young female rats reduced the lactogenic hormone content of the pituitary below that in normal rats. The proven ability of estrogen to increase the lactogen content of the pituitary remained unimpaired in rats which were given the estrogen together with thiouracil for 21 days. However, when rats received thiouracil alone for a 2-week preliminary period, the subsequent administration of estrogen plus thiouracil for 10 days failed to maintain even the normal level of lactogenic hormone.

The pituitary and thyroid weights of the thiouracil as well as thiouracil plus estrogen-treated rats were increased above the normal controls. The thyroid hypertrophy in the rats which received estrogen and thiouracil for 10 days following a preliminary treatment of thiouracil alone for 2 weeks was less than in the rats which received only thiouracil for the same period.

15839

A Brief Insulin Tolerance Test.*

G. J. Kronenberg and W. S. McCulloch.†

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Meduna *et al.*[1-3] by determining reducing substance (which is predominantly glucose) in venous blood at fasting and at 5, 10, 15, 20, 25, 30, 35, 45, 60, 90 and 120 minutes following the injection of 0.1 unit insulin per kg body weight, had found that the nadir was shallower and later in oneirophrenics than in normals, and had observed, in individual, normal records, a bump—*i.e.* a failure to fall or actual rise—before the nadir at which sympathetic counter-regulation initiates a rapid rise in blood-sugar peaking about the 45th minute. He had also found that throughout all of these changes the amount of reducing substances other than glucose was constant. The present work was begun in 1943 to increase the certainty of his separation and, by samples at the 12th and 17th minutes, to define the bump on the descending limb and finally, to construct a brief test for clinical use.

On the assumption that the effect of insulin in lowering the blood-sugar was proportional to its initial value, Meduna had expressed the observed decrease in percent of fasting value, and subsequent experience indicates that this is probably the best expression. He used colorimetric determination of alkaline ferricyanide reduced by tungstic acid filtrates from 0.1 ml of venous blood and this, by chance, gives mean fasting values of approximately 100 mg %, so that the mean

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† The authors wish to express their indebtedness for technical assistance to Miss Genevieve Mack, Miss Edith Laslo, and Miss Ellen Ridley.

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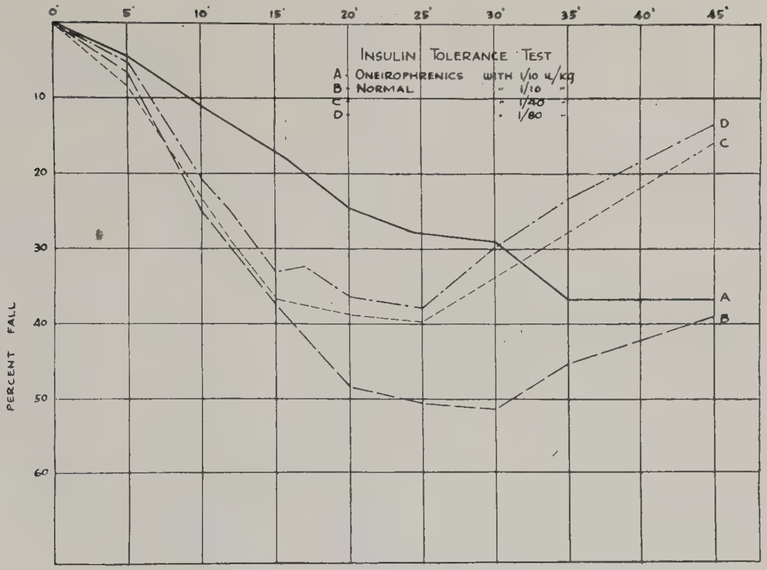

FIG. 1.

Insulin Tolerance Test in Oneirophrenics and Normals. Mean % fall of reducing substance of the blood in oneirophrenics (A) and in normals, showing early divergence and normal lack of dose sensitivity for the first 15 minutes, with doses of (B) 1/10, (C) 1/40, and (D) 1/80 u/kg.

TABLE I.

Subject	Dose	15′		20′		25′		30′	
A Oneirophrenic	1/10 u/kg	16.8	σ 7.6	24.3	σ 9.8	27.6	σ 4.4	29.8	σ 8.9
B Normals	1/10 ”	37.6	σ 10.1	48.4	σ 9.5	50.6	σ 8.8	51.7	σ 9.2
C ”	1/40 ”	36.8	σ 9.3	38.8	σ 9.0	39.8	σ 9.0	33.5	σ 9.6
D ”	1/80 ”	33.0	σ 7.0	36.3	σ 7.8	37.8	σ 7.6	29.0	σ 6.9
		t	P	t	P	t	P	t	P
AB	Fisher's t	7.75	<.01	8.2	<.01	7.0	<.01	8.1	<.01
AC	and prob.	7.5	<.01						
AD		6.87	<.01						

fall in percent of fasting is approximately equal to the mean fall in mg %. The present data was obtained and charted by his methods.

The following curves are based on the first 20 to 25 cases in which the diagnosis of the patient was certain, the health of the control assured, the technic faultless and the data available for computation at the time this material was organized. Cursory examination of more than 300 records has indicated no important exceptions.

Fig. 1A is based on Meduna's data and shows the mean blood-sugar level at various times during the first 45 minutes after injection of 0.1 unit insulin per kg body weight in his first 21 reported tests of oneirophrenics. Fig. 1, B, C, and D are the same for healthy controls receiving that standard dose and, ¼ and ⅛ of it respectively. These 3 normal curves are similar for the first 15 minutes and all fall far faster than the oneirophrenic at that time.

Table I gives the mean falls, the standard deviations, Fisher's "t" and the probability that the difference between the means is due to chance at the 15th, 20th, 25th and 30th minutes. From this it is clear that oneirophrenics differ significantly from normals through the 30th minute after which normals rise while some oneirophrenics continue to descend.

Table I also shows the lack of any reliable difference between the 3 groups of controls during the first 15 minutes, indicating that the rate of fall is independent of the dose

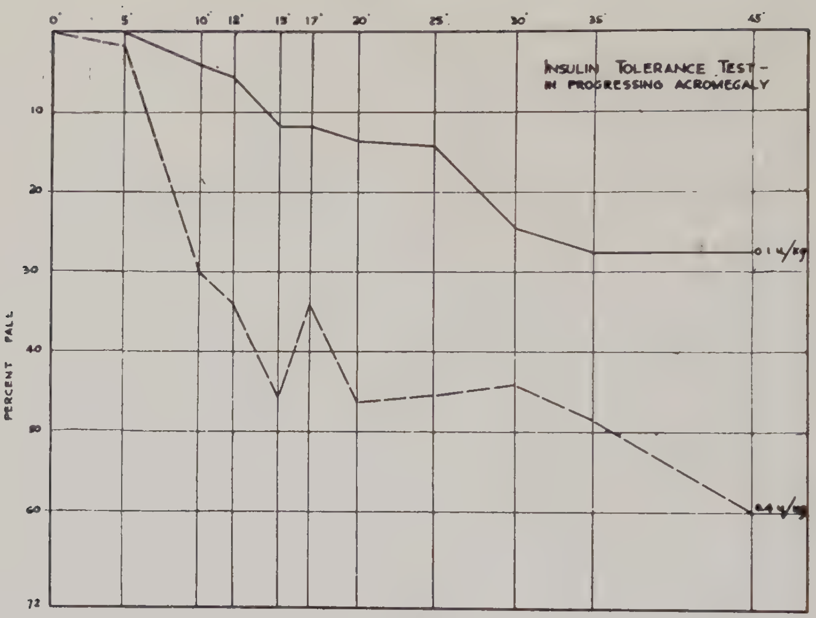

FIG. 2.
Dose Sensitivity in Acromegaly. Insulin tolerance curves in % fall of reducing substances of blood after 0.1 u/kg showing resistance but rapid fall and spike on descending limb after 0.4 u/kg.

which yet determines the nadir.

Finally, Table I shows that at the 15th minute the differences between oneirophrenics and normals receiving ⅛ as much insulin is still significant. In a few cases we have given 8 times the dose to oneirophrenics and found that, though it made the blood-sugar fall farther, it fell no faster. Obviously, therefore, in a clinical test one should use the early part of the test where a gross error in dose is insignificant.

Of many nondiabetic, medical and surgical conditions tested for increased resistance to insulin only one group of malignant hypertensives and the progressive acromegalic have shown resistance. The latter (Fig. 2) are interesting because the resistance can be exceeded by increasing the dose and then there may appear an exaggeration of the bump on the descending limb. Similar exaggerations have appeared following sundry operations on the brain.

The time at which this upward inflection occurs varies from the 12th to the 17th minute. A third of the bumps are actual spikes, a third are levels and a third are only decreased rates before the final, fast drop to the nadir. It is absent in less than one-tenth of normals and one-third of oneirophrenics, but it is frequently missed with 5-minute sampling and its temporal scatter averages it out in the mean curve except with the smallest dose. The mean value of the difference between the preceding and subsequent low and the mean highest value of the bump exceeds twice the mean error of the method and hence the probability that the bump is due to chance is negligible.

It is an increment in glucose. It is present in arterial blood. It occurs with all varieties of American insulin and with Danish insulin having no hyperglycemic factor. It is present in diabetis mellitus, Addison's disease, malignant hypertension, and after sundry insults to the brain. It is not prevented by terror or atropine, by sympathectomy or supradiaphragmatic vagotomy, by advanced disease of the liver or by splenectomy. It is not preceded or accompanied by any signs of autonomic change in EEG, EKG, blood pressure, respiration, pupillary diameter or dorsal skin potential. Presumably it is due to some as yet undiscovered mechanism counteracting some effect of insulin. Fortunately

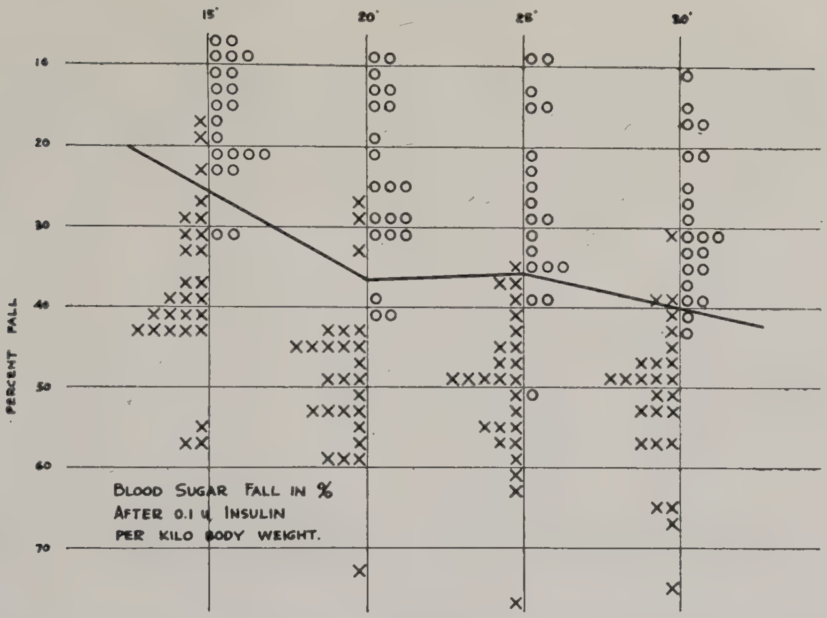

FIG. 3.
Scattergram for Use with Brief Insulin Tolerance Test. Scattergram of oneirophrenic, O, and normal, X, % fall from fasting of reducing substances of the blood.

it is not violent enough to prevent separation of oneirophrenics from normals.

Although in a given normal the blood-sugar may fall as fast and as far with an 80th as with a 10th unit insulin per kg body weight, the signs of so-called hypoglycemic shock—faintness, trembling, sweating, cardiac acceleration, and pupillary dilation—are minimal or absent. This would incline one to use the smaller dose, but as the law forbids sale of insulin at less than 20 units per cc one has to make up fresh sterile solutions for measurable doses, and many neurotics are resistant to it though they react normally to the regular dose. As the separation between oneirophrenics and normals is equally certain from the 15th through the 30th minute, only time is conserved by early sampling; but at the 15th minute the blood-sugar is falling 5 mg per minute in the normal which means that the timing must be accurate and venous return unobstructed. It is easier to note the exact time at which a sample is drawn than to draw it at a prescribed moment.

The following procedure, therefore, is recommended. The fasting sample is taken, then 0.1 unit insulin per kg body weight is injected intravenously. One or two samples are then taken between the 15th and 30th minutes and the time noted accurately. The patient may then be given sugar if desired. The total reducing substance is determined in each sample as noted above. If the fasting value differs significantly from 100 mg %, the falls are expressed in per cent of fasting; if not, they are left as mg %. Any such fall from fasting and the time after injection of insulin can then be compared with Fig. 3. This is a scattergram showing the distribution of oneirophrenics and normals on which is drawn one line running through those points at which a given value is as likely to be found in an oneirophrenic as in a normal. Inasmuch as the overlapping of the scattergrams is due to different mechanisms in the beginning and end of this interval, and no individual curves have been found to have more than one point across the line, 2 samples on either side of the line enhance the certainty of diagnosis. The chance of an erroneous diagnosis is, in fact, less than the product of chances for the 2 separately.

Summary. For the first 15 minutes the rate of fall in reducing substances in the blood expressed in per cent of fasting value is not much affected by decreasing the size of the intravenous dose of insulin below 0.1 u/kg or by increasing it in oneirophrenics. At 15 minutes the separation is statistically valid despite a bump of unknown significance on this descending limb of the curve. Therefore, it is possible, in patients suspected of oneirophrenia, to estimate the resistance to insulin with a statistical validity as great as can be obtained from much longer tests.

15840

Thiouracil in the Treatment of Leukemia.

O. C. Hansen-Pruss. (Introduced by W. A. Perlzweig.)
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It has been observed that patients with leukemia may show many of the clinical features of hyperthyroidism, such as nervousness, tachycardia, decreased cholesterol in the blood, and increased metabolic rate. Temporary improvement may result from the rational use of Lugol's solution.[1-3]

During the past 15 years, 175 patients with various types of leukemia have been studied in this clinic. The disease remained stationary or progressed slowly as long as the cholesterol level was above 150 mg %, but progressed rapidly when the cholesterol values fell below 100 mg %.

The cholesterol levels appeared to be influenced by the absolute number of extremely immature forms and not by the total number of circulating leukocytes. The cholesterol levels decreased as the number of immature forms increased, but in one group of 15 patients who progressed rapidly to a fatal end, with extensive systemic and visceral myeloblastic proliferation, the total counts were slightly elevated, normal or reduced, and the absolute number of myeloblasts small, yet the cholesterol levels were constantly below 100 mg %.

In remissions induced by X-ray therapy, the serum cholesterol values were uniformly above 150 mg %, and as the patients became resistant to the X-ray, the cholesterol levels fell.

There was no constant relationship between the basal metabolic rate, the serum cholesterol values and the prognosis.

It seemed desirable to determine whether the administration of thiouracil would increase the serum cholesterol levels and improve the patient's symptoms.

Case 1. S.K., white female, age 29 years with chronic myelogenous leukemia. The disease was apparently in its second year when it was recognized in 1940. Between October 1940 and June 1945, she received several series of X-ray treatments. These were given by spray and locally over the spleen. (These treatments totalled 1100 R. by spray and 1950 R. over the spleen). She experienced several striking clinical and hematological remissions. The last X-ray treatment was on June 5, 1945. The subsequent events are summarized in Table I.

This patient received 1.5 g of thiouracil a day over a period of 60 days, or a total of 90 g. She tolerated the drug quite well. She had an irregular, intermittent fever which was present before the administration of thiouracil was begun and persisted thereafter. At first, she seemed clinically improved; she felt stronger and the hemoglobin and red cell levels rose temporarily. The circulating leucocyte count fell precipitately during the first 24 hours after the drug was given, but

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TABLE I.
Temporary Drop in the White Count After Thiouracil in Chronic Myelogenous Leukemia.

Date	Thiouracil g/day	WBC (thous.)	Mature Myeloid C. %	Immature Myeloid C. %	Hgb., g	RBC (mill.)	B.M.R. %	Serum cholest., mg %	Bone marrow WBC (thous.)	Bone marrow Myelocytes, %	Bone marrow Myeloblasts, %
9/10/45	—	400.0	45	35	6.0	3.0	+14	165	274	33	17
11	1.5	*430.0	—	—	6.4	2.0	—	—	—	—	—
12	”	*109.0	50	44	6.3	—	—	—	—	—	—
13	”	130.0	—	—	—	—	—	—	—	—	—
14	”	134.0	59	31	7.2	3.1	—	175	280	42	22
21	”	170.0	60	31	7.3	2.3	+17	210	—	—	—
28	”	155.0	39	46	8.3	2.5	+24	220	—	—	—
10/ 3	”	146.0	45	50	8.6	2.5	+ 8	212	—	—	—
10	”	120.0	40	52	10.6	3.5	+10	210	—	—	—
11/12	”	283.0	32	57	7.3	2.3	+14	145	450	28	46

* Checked repeatedly.

there was no corresponding quantitative change in the bone marrow. Furthermore, there was no appreciable change in the maturation pace of the bone marrow elements. The drug did not affect decisively the red cells, hemoglobin or platelets. The basal metabolic rate did not fall to any important extent and the cholesterol level did not change. She grew gradually worse, lost weight, became steadily weaker and the spleen enlarged progressively and rapidly. In mid-November, she developed purpura (platelet count 100,000). It is obvious that thiouracil had failed to decrease the myeloid hyperfunction and leukocytosis and to influence the rate and degree of maturation of the myeloid elements. The patient became resistant to X-ray therapy, failed rapidly and died 7 months later.

Case 2. D.C.L., white female, age 49 years. Chronic myelogenous leukemia diagnosed in 1941. Between July 1941 and July 1945 she received a series of X-ray treatments by spray and over the spleen. (A total of 1150 R. over the spleen and 550 R. by spray). The induced hematological and clinical remissions were satisfactory. The last X-ray treatment before thiouracil was given on January 11, 1945. The subsequent developments are shown in Table II.

This patient received a total of 26.7 g of thiouracil in 15 days. She tolerated the drug quite well and remained afebrile. There was no significant quantitative or qualitative change in the peripheral white blood cell picture or in the bone marrow pattern. The red cell count and hemoglobin value remained unchanged throughout the period of observation. There was no demonstrable increase or depression of red cell formation, nor the platelets influenced. It is apparent that thiouracil, in this patient, failed to depress the basal metabolic rate since at the end of the second week the basal metabolic rate had risen to +47% and the serum cholesterol had fallen materially. A partial remission in the disease was induced by X-ray treatments later on.

Case 3. B.E.D., white male, age 27 years, suffering from myelogenous leukemia of 3

TABLE II.
Negative Effect of Thiouracil on Total White Count in Myelogenous Leukemia.

Date	Thiouracil g/day	WBC (thous.)	Mature Myeloid C. %	Immature Myeloid C. %	Hgb., g	RBC (mill.)	B.M.R. %	Serum cholest., mg %	Bone marrow WBC (thous.)	Bone marrow Myelocytes, %	Bone marrow Myeloblasts, %
7/ 3/45	Received 3 blood	500	38	47	6.4	2.0	+31	205	458	32	6
5	transfusions	346	67	26	8.0	2.2	—	—	—	—	—
9	—	520	50	49	9.0	2.9	—	200	—	—	—
10	1.6										
12	"	515	52	45	9.8	2.9	—	—	—	—	—
15	"	370	36	62	9.7	2.7	—	—	—	—	—
16	1.9										
17	"	445	37	63	8.8	3.1	+32	205	—	—	—
24	"	378	43	56	9.7	3.3	+47	120	383	50	7
25	X-ray begun										
8/ 1		85	81	17	9.1	3.1	—	200	—	—	—

TABLE III.
Striking Failure of Thiouracil in Lowering the Basal Metabolic Rate in Chronic Myelogenous Leukemia.

Date	Thiouracil g/day	WBC (thous.)	Mature Myeloid C. %	Immature Myeloid C. %	Hgb., g	RBC (mill.)	B.M.R. %	Serum cholest., mg %	Bone marrow WBC (thous.)	Bone marrow Myelocytes, %	Bone marrow Myeloblasts, %
8/ 6/45	—	56.0	*61	36	6	1.8	+70	83	250	40	15
7	1.6										
9	"	26.2	*58	32	6	2.0	—	—	—	—	—
13	"	30.0	*63	20	6.9	1.9	+89	70	—	—	—
19	"	37.5	*47	41	6.8	1.8	—	—	215	†38	28

* 25% or more were basophiles.
† Many basophiles.

years duration. He had received a series of X-ray treatments with temporary improvement. The last X-ray treatment was given in December 1944, 8 months before thiouracil was tried. (A total of 1350 R. by spray and 1075 R. over the spleen). The result of thiouracil treatment is summarized in Table III.

It is obvious that thiouracil did not affect the total circulating white cell mass, the bone marrow pattern, the basal metabolic rate or the serum cholesterol. The erythroblastic system was not influenced. This patient's high basophile count was probably due to the prolonged X-ray treatments. Thiouracil, again, did not influence the hemoglobin, red cell or platelet values. This patient expired two weeks later with profuse internal and external hemorrhages.

Case 4. E.M.G., white female, age 40 years. This patient had noticed repeated bleeding into the skin, from the gums, and a progressive abdominal discomfort over a period of one year. She was admitted to the surgical service and the disease was recognized as chronic myelogenous leukemia with striking splenomegaly. She received a series of X-ray treatments by the spray method and over the spleen. (Spray 100 R.; spleen 450 R.) Since she had not received any previous treatments, the patient was considered a suitable subject to test the effect of thiouracil on the X-ray-induced remission in myelogenous leukemia. These observations are shown in Table IV.

It is apparent that thiouracil did not prolong or modify the X-ray-induced remission. She received 359.3 g of the drug in 10 months. The variations in the metabolic rate and serum cholesterol are rather striking, but inconclusive.

Two other patients with chronic myelogenous leukemia were given thiouracil to test the effect of this drug on X-ray-induced remissions. The results were analogous to that described above. It is obvious that thiouracil does not prolong or modify the remissions induced by X-ray treatments.

Thiouracil was given to one patient with an untreated myeloblastic leukemia. He re-

TABLE IV.
Effect of Thiouracil on the Duration of X-Ray Induced Remission in Chronic Myelogenous Leukemia.

Date	Thiouracil g/day	WBC (thous.)	Mature Myeloid C. %	Immature Myeloid C. %	Hgb., g	RBC (mill.)	B.M.R. %	Serum cholest., mg %	Bone marrow WBC (thous.)	Bone marrow Myelocytes, %	Bone marrow Myeloblasts, %
6/14/45	—	710	41	54	8.3	2.5	+70	—	575	27	2
	X-ray Px										
	Spray 100										
	Spleen 450										
	R.U. 550										
21	0.6										
26	,,	35.0	63	26	10.5	3.4	—	—	—	—	—
8/29	0.9	9.0	70	1	14.2	4.7	— 6	225	—	—	—
9/ 6	,,	11.0	72	2	14.1	4.9	—	210	—	—	—
27	1.2 to 1.5	6.5	66	2	14.4	4.9	+ 2	320	—	—	—
10/11	,,	9.2	76	6	15.6	4.7	+16	230	—	—	—
11/ 8	,,	7.7	72	9	16.0	5.2	— 1	238	—	—	—
2/ 8/46	,,	115.0	64	33	12.6	4.0	+11	190	—	—	—
3/11	,,	176.0	48	36	14.3	4.5	+33	160	—	—	—
4/ 3	,,	166.0	70	26	13.1	4.2	+19	170	397	28	1

ceived 1.5 g a day. There was no change in the peripheral white cell picture, qualitatively or quantitatively, nor did the bone marrow pattern change. This patient expired on the 15th hospital day of thiouracil treatment.

Discussion and Conclusions. Thiouracil was administered to 6 patients with chronic myelogenous leukemia, and to one patient with an acute myeloblastic leukemia. The dosage averaged 1.5 g a day and was well tolerated by every patient. One patient (case 4) received a total of 359.3 g of the drug over a 10-month period. The drug had no effect on the total circulating white count, nor on the differential white cell picture, red cell elements, hemoglobin or platelet values or the bone marrow pattern.

Recently, and after completion of this study, one observer reported that he had tried thiouracil in doses of 3 mg daily in a patient with myelogenous leukemia without appreciable results.[4]

Thiouracil did not influence the metabolic rate of leukemic patients materially when given in a dose of 1.5 g daily. This was true whether it was administered over a period of 2 weeks, 2 months or 10 months. The serum cholesterol rose in only 2 patients (cases 2 and 4), and this rise was only transitory. In one patient (case 3) the serum cholesterol continued to fall, the basal rate steadily rose despite the administration of thiouracil. In one patient reported (case 4), and in 2 others, this drug failed to prolong or modify the remission induced by irradiation. Thiouracil given in the dosage reported in this series had no demonstrable effect on the myeloid dysfunction, basal metabolic rate, the serum cholesterol level or the bone marrow pattern of patients with myelogenous leukemia.

Thiouracil, furthermore, did not modify the clinical course of the disease. It seems certain therefore, that this drug has no value as an adjunct in the treatment of myelogenous leukemia.

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[4] Limarzi, L. R., Kulasavage, R. J., and Pirani, C. L., *J. Hem. (Blood)*, 1946, **1**, 426.

15841

Effects of Liver Feeding on Growth and Ovarian Development in the Hyperthyroid Rat.*

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It has been demonstrated that excessive doses of thyroxin or desiccated thyroid cause anestrus and loss of body and ovarian weight in the adult rat and prevent gonadal development in the young animal. The deleterious effects in the adult rat according to Drill *et al.*[1] can be prevented by the administration of increased amounts of thiamine or yeast. Such nutrients are without effect, however, in the young animal.[2] It appears, therefore, either (1) that the young rat is more susceptible to the toxic effects of thyroid insofar as gonadal physiology is concerned or (2) that excessive thyroid feeding may precipitate a deficiency of some nutrient other than the above necessary for gonadal development in the immature rat. In the present experiment attempts were made to prevent by dietary means the failure of ovarian development in the immature thyroid-fed rat.

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* The subject matter of this paper has been undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The opinions or conclusions contained in this report are those of the author. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

[1] Drill, V. A., Overman, R., and Leathem, J. H., *Endocrinology*, 1943, **32**, 327.

[2] Ershoff, B. H., *Endocrinology*, 1945, **37**, 218.

Procedure and Results. Three basal rations were employed in the present experiment: diets A, B and C. Diet A was a purified ration containing the B complex factors in synthetic form only. Diets B and C were similar in composition but contained yeast or desiccated liver in addition to the synthetic vitamins. All 3 rations were supplemented with 0.0 and 0.5% U.S.P. desiccated thyroid. Seventy-eight female rats of the Long-Evans strain were weaned at 21 to 23 days of age and litter mates divided as far as possible among the experimental groups listed in Table I. Animals were kept in metal cages with raised screen bottoms to prevent access to feces, and sufficient food was administered to assure *ad lib* feeding. Subsequent to the 40th day of feeding, vaginal smears were taken daily of all rats. Surviving animals were autopsied on the 60th day of feeding; ovaries were weighed and fixed in 10% formol, and sections prepared and stained with hematoxylin and eosin.

Results are summarized in Table II. In agreement with earlier findings[4] the mortality rate of thyroid-fed rats was greater on the synthetic diet than on rations containing yeast. Diets containing liver were similarly effective in prolonging survival. A significant difference in body and gonadal weight was observed in thyroid-fed rats on the various diets employed. On diets A and B both body and ovarian weight was significantly less in thyroid-fed rats than in animals fed a similar diet with thyroid omitted; and ovaries of the thyroid-fed animals resembled in microscopic appearance those of the immature rat. On diet C, however, no significant differences in body and gonadal weight were observed between animals fed thyroid and those on a thyroid-free ration; and histologically the ovaries of thyroid-fed rats appeared normal in all respects. In the absence of dietary thyroid body and ovarian weights did not differ significantly on any of the diets employed; and histologically ovaries appeared normal in all groups. Data obtained from vaginal smears check with the above findings. On diets A and B thyroid-fed rats exhibited constant leucocytic smears subsequent to the 40th day of feeding in contrast to the regular estrus cycles obtained on diet C. In the absence of dietary thyroid estrus cycles were regular on all diets. Subsequent work indicates that the ovaries of thyroid-fed rats raised to maturity on diet C were normal functionally as well as histologically. When bred to normal males after the 60th day of feeding, such animals while still on the same diet gave birth to apparently normal young.

TABLE I.
Composition of Experimental Diets.

Dietary component	Diet A	Diet B	Diet C
Yeast*	0.0	10.0	0.0
Liver†	0.0	0.0	10.0
Vitamin Test Casein‡	22.0	22.0	22.0
Salt Mixture§	4.5	4.5	4.5
Sucrose	73.5	63.5	63.5

When thyroid was fed it was included in the above diets at a level of 0.5%, replacing an equal amount of sucrose.‖

To each kg of the above diets were added the following synthetic vitamins: thiamine hydrochloride 72 mg, riboflavin 9 mg, pyridoxine hydrochloride 15 mg, calcium pantothenate 67.2 mg, nicotinic acid 60 mg, 2-methyl-napthaquinone 5 mg, and choline chloride 1.2 g. Each rat also received 3 times weekly the following supplement: cottonseed oil (Wesson) 500 mg, alpha-tocopherol 1 mg, and a vitamin A-D concentrate¶ containing 50 U.S.P. units of vitamin A and 5 U.S.P. units of vitamin D.

* Brewer's Type Yeast No. 200, Anheuser-Busch, Inc., St. Louis, Mo.

† Whole Dried Liver Powder, Armour and Co., Chicago, Ill.

‡ Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

§ Sure's Salt Mixture No. 1.[3]

‖ Thyroid Powder, U.S.P., Armour and Co., Chicago, Ill.

¶ Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per gram.

Available data indicate that the factor in liver responsible for the above effects is not one of the major B vitamins. Doubling the B vitamin content of diet A was without significant effect on the body or gonadal weight of thyroid-fed rats. Individual supplements

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[3] Sure, B., *J. Nutrition*, 1941, **22**, 499.

[4] Ershoff, B. H., and Hershberg, D., *Am. J. Physiol.*, 1945, **145**, 16.

TABLE II.
Effects of Thyroid Feeding on Body and Gonadal Weight.

Dietary group	% thyroid	No. of animals	Avg body wt Initial g	Avg body wt On 60th feeding day* g	Avg ovarian wt* mg
A	.5	20	43.7	116.5 ± 6.7 (4)	23.6 ± 2.2
B	.5	20	42.9	133.5 ± 7.8 (14)	27.8 ± 4.1
C	.5	20	43.3	202.3 ± 8.1 (15)	72.3 ± 7.1
A	.0	6	42.7	198.7 ± 4.1 (6)	53.1 ± 3.3
B	.0	6	43.1	186.6 ± 6.7 (6)	56.8 ± 4.7
C	.0	6	42.7	197.5 ± 4.4 (6)	59.4 ± 5.1

The values in parentheses indicate the number of animals which survived of which this is an average.

* Including standard error of the mean calculated as follows: $\sqrt{\frac{\epsilon d^2}{n}} \ / \ \sqrt{n}$

where "d" is the deviation from the mean and "n" is the number of observations.

of thiamine, riboflavin, pyridoxine, calcium pantothenate, nicotinic acid, inositol, *p*-aminobenzoic acid, biotin or folic acid were similarly ineffective.† Doubling the salt content of diet A or administering an additional 10% casein or 10% wheat germ‡ at the expense of an equal amount of sucrose were also without effect. Only liver of all substances tested gave a positive response in the thyroid-fed rat. Preliminary results indicate that the factor in liver responsible for the above effects is heat stabile.

Discussion. Evidence is accumulating that in addition to the major nutrients, substances are present in our diet which may be required in increased quantities during conditions of stress. Such factors are apparently dispensable under normal conditions or their requirements are so small they may readily be met by amounts present in the diet or through the synthetic activity of the intestinal flora or the animals' own tissues. Certain drugs or other "stress factors" may, however, increase requirements for these substances to the extent that deficiencies occur, manifest by retarded growth or tissue pathology, and preventable by the administration in appropriate amounts of the missing nutrient. Present findings indicate that thyroid feeding is such a factor. Although liver did not counteract other manifestations of hyperthyroidism, such as a hypertrophied ventricular wall, reduction in the creatine content of the heart or an increased B.M.R.,[5] it did counteract completely the retardation of growth and inhibition of ovarian development observed on all other rations when thyroid was fed.

Findings indicate that liver contains an organic factor other than thiamine, riboflavin, pyridoxine, calcium pantothenate, nicotinic acid, inositol, *p*-aminobenzoic acid, biotin or folic acid, a substance not present in significant amounts in wheat germ, casein or yeast, but required for normal growth and ovarian development in the immature thyroid-fed rat.

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† Supplements were added to diet A in the following amounts per kg of diet: thiamine hydrochloride 3.6 g, riboflavin 450 mg, pyridoxine hydrochloride 750 mg, calcium pantothenate 3.36 g, nicotinic acid 3.0 g, inositol 10.0 g, *p*-aminobenzoic acid 10.0 g, biotin 1.0 mg, and folic acid 10.0 mg. We are indebted to Dr. Stanton M. Hardy of the Lederle Laboratories, Pearl River, New York, for the folic acid employed in the present experiment. The remaining B vitamins were obtained from Merck and Co., Rahway, N.J.

Subsequent work indicates that individual supplements of ascorbic acid and the fat-soluble vitamins were also ineffective. These were added to diet A in the following amounts per kg of diet: ascorbic acid 10 g, vitamin A 100,000 U.S.P. units, vitamin D 25,000 U.S.P. units, and 2-methyl-napthaquinone 100 mg. Alpha-tocopherol was administered as a daily supplement at a level of 5 mg per rat.

‡ Defatted Wheat Germ, Vio-bin Corporation, Monticello, Ill.

[5] Ershoff, B. H., in press.

Summary. The administration of liver completely counteracted the retardation of growth and inhibition of ovarian development observed in immature rats fed toxic amounts of thyroid. Wheat germ, yeast, or increased amounts of salt mixture, casein, thiamine, riboflavin, pyridoxine, calcium pantothenate, nicotinic acid, inositol, *p*-aminobenzoic acid, biotin or folic acid were ineffective in this regard. The suggestion is made that liver contains some factor other than the above required for normal growth and ovarian development in the immature thyroid-fed rat.

15842

Some Pharmacological Characteristics of Bacitracin.*

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Bacitracin, an antibiotic discovered by Meleney and co-workers[1] in the filtrate of surface cultures of the Tracy strain of *B. subtilis*, has become of increasing interest, particularly because of its potential value in the treatment of penicillin-resistant infections. Because of this increased interest, the following preliminary data on the toxicological and pharmacological properties of the antibiotic are presented.

Acute toxicity experiments. Fresh bacitracin[†] solutions used for oral and intraperitoneal administration contained the dose in 1 cc of water; solutions for intravenous and subcutaneous injections contained the dose in 0.25 to 0.50 cc per 20 g of mouse, or 50 g of rat. All samples gave clear solutions at pH 6 to 7 except No. 187, which dissolved at pH values of 5 to 6. For oral or subcutaneous administration, fine suspensions were administered because of the large doses required. Solutions of earlier samples of bacitracin were brown, but more highly purified later samples, illustrated by No. B-100, were almost colorless.

Experiments were performed with male, white, Rockland Swiss mice weighing 20 to 25 g, and male white rats of the Sherman strain maintained on a stock diet with free access to food and water at all times. Surviving animals were observed for a period of at least 7 days after acute or chronic dosing.

Results of the acute toxicity experiments are shown in Table I. Comparison of the first 4 samples, all administered intraperitoneally, indicates that the toxicity varies independently of the activity. (The fourth sample, No. B-100, is much less toxic than the other 3 and is representative of the batches now being produced). In this connection it may be noted that inactivation of the first sample under mild conditions (pH 7.0 at 37° for 8 days) did not alter the toxicity of the preparation. The LD_{50}, 200 mg per kg, did not differ significantly from the value of 240 mg per kg obtained with the active sample. Neither the scattering of the deaths, nor the toxic signs, nor the pathological lesions in mice differed appreciably

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[*] The work described in this paper was done under a contract between the Office of the Surgeon General and Columbia University. Administration of the contract was directed by Dr. Frank L. Meleney.

[1] Johnson, B. A., Anker, H., and Meleney, F. L., *Science*, 1945, **102**, 376.

[†] We are indebted to Dr. John T. Goorley of the Ben Venue Laboratories for the bacitracin.

TABLE I.
Acute Toxicity of Bacitracin in Mice and Rats.

	Sample No.	Animal	Mode of administration	Activity of sample U per mg	No. of animals between LD_{16} and LD_{84}	LD_{50} mg per kg	LD_{50} units per kg	σ	Stand. dev. LD_{50}
1	187	mice*	I‡	35	170	240	8,500	3,800	410
2	188	,,	,,	13	90	650	8,500	2,000	300
3	203	,,	,,	21	113	360	7,500	2,400	320
4	B-100 new process	,,	,,	30	80	500	15,000	4,900	770
5	187 inactivated	,,	,,	0	40	200 σ = 50 $SD.LD_{50}$ = 11			
6	203	,,	Iv§	21	75	360	7,500	1,300	210
7	203	,,	S‖	21	75	2,500	52,000	20,000	3,300
8	208WI	,,	,,	24	70	1,300	31,000	7,000	1,200
9	187	rats†	I‡	35	49	190	6,700	2,500	500

* The majority of deaths occurred during the first 24 hours, with large numbers dying during the second 24 hours, and occasional deaths thereafter.

† All deaths occurred within the first 24 hours.

‡ I = intraperitoneal.

§ Iv = intravenous.

‖ S = subcutaneous.

from those observed with the active material. Since the toxicity of the sample was not altered by destruction of the active principle, it seems reasonable to assume that further purification of bacitracin will lead to a lowered toxicity. The third sample (203) gave identical LD_{50}'s after intravenous and intraperitoneal injections. The acute subcutaneous and oral toxicities of bacitracin were much lower than the intraperitoneal toxicity: for example, sample No. 203 was about 7 times as toxic when given intraperitoneally as when given subcutaneously. There was no direct comparison for the eighth sample (208 WI) but here again the subcutaneous toxicity represented by the LD_{50} was notably low. Given orally in amounts increasing up to 3.75 g per kg or 131,000 units per kg, sample No. 187 failed to produce death. At 7.5 g per kg 2 mice out of 5 died. These deaths may have been influenced by the high osmotic concentration of the solutions administered. The oral and subcutaneous findings are similar to those observed by Molitor *et al.*[2] in a study of streptomycin.

The acute intraperitoneal toxicity of sample 187 was significantly greater in rats than in mice. There were no deaths subsequent to the first 24 hours after the administration of the drug to rats. Further, renal lesions, described below, were striking in mice but were minimal in rats. The intravenous toxicity of bacitracin appeared to be greater in rats than in mice. Sudden, convulsive deaths could be produced regularly at doses as low as 4000 units per kg of sample No. 187 intravenously.

Signs of acute toxicity were similar regardless of the sample of bacitracin used. Following intraperitoneal injection of lethal doses, mice exhibited hyperirritability and clonic convulsions followed by an increasingly severe depression. Central respiratory paralysis appeared to be the cause of death in both mice and rats. Similar effects were produced more rapidly by intravenous injections. No acute toxic effect other than apathy was noted after oral or subcutaneous administration of the impure antibiotic. Complete blood counts were made in all mice prior to, and serially in surviving mice for 8 days after the intraperitoneal injection of B-100. Although large doses, grouped around the LD_{50}, were given, no abnormalities in blood morphology were observed.

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[2] Molitor, H., Graessle, O. E., Kuna, S., Mushett, C. W., and Silber, R. H., *J. Pharm. Exp. Ther.*, 1946, **86**, 151.

Chronic toxicity experiments. Groups of 25 mice were given bacitracin (No. 187) subcutaneously at doses of 2, 20, and 50 units per 20 g mouse[‡] every 4 hours for 5 days. The total dose administered during the 5-day period was 3,000, 30,000 and 75,000 units per kg of mouse. None of the animals died during the period of treatment nor in the following 7 days. The animals appeared normal and made average weight gains of 5% of body weight during the 5 days of treatment and 10% during the ensuing 7 days, regardless of the dose given.

Rats weighing 80 to 90 g were given 6,000 units per kg of bacitracin intraperitoneally. One week later surviving animals were divided into 2 groups of 10 each. Animals in the first group were given, subcutaneously, 650 units per kg per day, 5 days of each week for 13 doses followed by 12 additional doses intraperitoneally. Animals in the second group were maintained on the same schedule but the chronic doses were doubled. Rats in the first and second groups received a total of 22,250 and 38,500 units of bacitracin per kg, respectively. With the exception of local areas of alopecia at the site of injection all animals appeared grossly normal. During the 5 weeks of treatment, animals of the first group grew from an average of 88 to 177 g. Those in the second group grew from 84 to 150 g, suggesting that the larger doses impeded growth to a slight extent.

Pathological changes in mice and rats following single or repeated doses. Gross examination of the organs following acute deaths showed congested splanchnic vessels and emphysematous lungs. Mice which survived large doses for more than 24 hours lost weight and exhibited enlarged, edematous, ischemic kidneys. Kidney involvement appeared maximal 3 to 5 days after intraperitoneal injection of bacitracin, and then appeared to regress. With the exception of adrenal enlargement, remaining organs were normal grossly. At the site of subcutaneous injection, induration, congestion and hemorrhage were often found with the early preparations; product B-100, manufactured by a new process, caused considerably less irritation.

Histologically, renal damage in the mouse was striking. With moderate involvement, the cytoplasm of cells of the convoluted tubules was filled with fine eosinophilic granules, particularly in the free portion of the cell; in places it appeared as though this portion of the cell was being cast into the lumen. The loops of Henle, particularly the descending limbs, were filled with deeply staining eosinophilic material usually in the form of casts or globules. Similar material was also present in the collecting tubules. The loops of Henle and collecting tubules were markedly dilated. With severe involvement there was tubular necrosis so that identification of the individual tubule was difficult. There was necrosis of the convoluted tubules which appeared to involve the distal convoluted tubules and, occasionally, the proximal tubules. The lumina were filled with necrotic cells, nuclear debris and calcium particles. Similar material was found in the loops of Henle and collecting tubules. Rarely a tubule was calcified. Bowman's space was distended with coagulum but the glomerular tuft proper appeared uninvolved. Mice in which appreciable tubular necrosis was found were either dying or were sacrificed when moribund.

Mice which survived 6 days after administration of the drug showed marked dilation of the collecting tubules and, to a less extent, the loops of Henle. The collecting tubules contained eosinophilic globoid bodies or casts which completely filled the lumen and had the appearance of coagulated serum. At times basophilic bodies were found. Tubular calcification was not frequent. After 10 to

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[‡] This may be assumed from the work of Meleney *et al.* to represent 1, 10, and 25 times the therapeutic dose.

12 days a less pronounced picture was observed. There were small depressed subcapsular areas composed of collapsed tubules, and a rare atrophic glomerulus. Cystically dilated tubules and collections of monocytic cells about vessels were seen. Some of the tubules were lined with basophilic cells and mitosis was occasionally observed. The nuclei of the tubular cells varied considerably in size and staining quality. With repeated subcutaneous doses varying from 2-50 units per 20 g mouse, renal lesions observed one week after the final injection were inconspicuous, consisting for the most part of occasional tubular casts. Necrotic tubules were infrequent.

The thyroids appeared moderately hyperplastic.

In the rat renal lesions were minimal and necrosis of cells rare.

Other pharmacological data. The best available preparation of bacitracin (No. B-100) was used in gathering the following data. A 5% solution containing 1500 units per cc was found, by means of the freezing point depression, to be approximately isotonic with frog-Ringer solution. The contractility and rhythmicity of the frog's heart perfused by the Straub technic were not altered by the addition of 15 units of bacitracin per cc of frog-Ringer solution. Higher concentrations often caused diminished contractility and progressive slowing.

Tests with the isolated guinea pig ileum indicated that bacitracin contains a minute amount of a histamine-like substance; for example, 100 mg or 3,000 units of preparation B-100 contained the equivalent of approximately 3 μg of histamine diphosphate. Bacitracin-induced contractions were essentially abolished by first adding 1 cc of 1-100,000 pyribenzamine hydrochloride to the 50 cc bath used in the experiments.

Intravenous injection of bacitracin solution into the dog, anesthetized by intravenous pentobarbital, caused an acute transient fall of blood pressure immediately followed by an equally transient rise; for example, 10 mg or 300 units of lot B-100 per kg body weight caused a fall of 30-40 mm of Hg followed by a rise of 10-20 mm of Hg above the pre-injection level of the blood pressure. Each effect lasted only a few seconds. Either benadryl or pyribenzamine essentially abolished the action of equidepressor doses of histamine diphosphate, but neither altered the depressor effect of bacitracin. Hence, histamine is not the cause of the depressor effect. This depressor effect, unlike that of acetylcholine, was not prevented by atropine sulfate. The pressor phase of the diphasic blood pressure response and the vagal heart beats associated with it disappeared after the injection of pyribenzamine.

Summary. The acute, intraperitoneal toxicity of bacitracin concentrates was greater in rats than in mice. The subcutaneous LD_{50} in mice was 4-7 times the intraperitoneal LD_{50}. Lethal results were produced only with immense oral doses. Damage to the renal tubules, with tubular necrosis after very large doses, was observed in mice whereas in rats there was little kidney involvement. The toxicity of bacitracin, as measured by the LD_{50}, appeared to be independent of the antibiotic activity. Concentrates of the antibiotic contained a minute amount of an histamine-like substance which was not responsible for the definite depressor effect caused by the intravenous injection of 10 mg (300 units) per kg into the dog.

We are indebted to Professor Harry B. van Dyke for his kind interest and active participation in this work.

15843

Pathogenicity of the Tubercle Bacillus.*

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The factors responsible for the ability of *Mycobacterium tuberculosis* to produce disease in a susceptible host have long been enigmatic. The criteria commonly employed to judge the probable virulence of many other pathogenic bacteria have not been applicable in this instance. For example, although dissociation occurs in cultures of tubercle bacilli,[1] smoothness or roughness of colonial form does not provide a satisfactory basis for predicting the activity of this organism in the tissues of a susceptible species of host.[2] It is mystifying indeed that an attenuated bacterium such as the B.C.G. (Bacillus Calmette-Guérin) should possess, so far as we know, all the properties of the virulent tubercle bacillus except the ability to produce a progressive generalized infection. At the site of injection it induces the typical tuberculous lesion, and from this area viable bacilli may be cultured after many months.

It is difficult to deduce from available information whether the virulent tubercle bacillus possesses some cytoplasmic constituent analogous to the somatic antigens associated with virulence in many other bacterial species. One of the major functions of such substances in permitting organisms to multiply in tissues is the phagocyte-repellent effect. No such effect is apparent in the case of *M. tuberculosis*, for virulent as well as attenuated organisms are readily taken up by monocytic phagocytes. Its pathogenicity may conceivably depend upon a metabolic feature of the bacterial cell, the virulent organism being better able to propagate in the environment supplied by viable tissues. For this view also evidence is lacking.

Should virulence depend in part upon a specific cellular antigen, it seems a rational probability that the acquisition of immunity will also be related to this substance, for in the final analysis acquired resistance means the ability of the body specifically to "neutralize" the virulence mechanism of the bacterium. A familiar case in point is the protection provided by the antibody directed against the capsular S.S.S. of *D. pneumoniae.* If, on the other hand, virulent tubercle bacilli are distinguished by a metabolic trait which favors their growth in the tissues, then it is quite possible that no ordinary immunologic device with which we are familiar will divulge the nature of acquired resistance to them.

Several years ago we undertook a study designed to reveal whether some one antigenic component of *M. tuberculosis* may be responsible for inducing acquired resistance, and as a corollary, whether this factor would enable us to demonstrate, on an immunologic basis, the dissociation or identity of resistance and allergy in this disease.[3]

Attempts to induce the resistant state in the absence of allergy have so far been unsuccessful. A good deal of attention has been devoted to the polysaccharide of the bacillus in this connection. This substance however is haptenic, and its reconversion to a complete antigen has not proved satisfactory, so that a fair test of its immunizing ability has not been possible. However, we have found that when this substance was injected daily in amounts up to 15 mg to guinea pigs throughout a 10-week course of immunization with B.C.G., and then for 20

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* Carried out with the aid of grants from the California Tuberculosis and Health Association and the National Tuberculosis Association.

† With the assistance of Dr. Carl M. Johnson, Mrs. Jessie Billon and Mr. Waldo H. Hanns.

[1] Petroff, S. A., and Steenken, W., Jr., *J. Exp. Med.*, 1930, **51**, 831.

[2] Smithburn, K. C., *Am. Rev. Tuberc.*, 1937, **36**, 637.

[3] Raffel, S., *Stanford Med. Bull.*, 1943, **1**, 209.

weeks subsequent to infection with virulent organisms ($H_{37}Rv$), it failed to antagonize the acquired resistance produced by the B.C.G.[4] It appears then that acquired immunity to *M. tuberculosis* is not related in any major degree to an antibody type of response to this particular bacillary antigen. The purified phosphatide, the wax, and the protein have also been employed as vaccinating materials, and these have failed to induce resistance to the bacillus of origin.[5]

More success has attended our studies of the possible relationship of tuberculous allergy to resistance. It has been appreciated for many years that this hypersensitive state is a response to the protein of the organism. Peculiarly enough, however, the tuberculin-type of allergy cannot be initiated by the use of the bacillary protein in isolated form, although the substance has marked antigenic properties and on injection into normal animals regularly induces humoral antibodies and the anaphylactic state. We have found that the delayed type of tuberculin hypersensitivity can be established in the absence of tubercle bacilli by employing the chloroform extractable "wax" of the bacillus. Such wax contains a small proportion of protein, and when injected into normal guinea pigs it regularly establishes good levels of delayed reactivity to Old Tuberculin.[5] The application of several criteria for the tuberculin-type of hypersensitivity, including vulnerability of such sensitized tissues to tuberculin in tissue culture, and failure to transfer the sensitivity passively via serum, have demonstrated that this is true tuberculous allergy. Tests on a limited number of guinea pigs have indicated that animals rendered hypersensitive by this means do not possess acquired resistance to tuberculous infection. This observation has convinced us that allergy and acquired immunity are immunologically distinguishable.

Experimental. Our more recent efforts to isolate a resistance-inducing factor from the bacillus has led us to investigate antigens of the "Boivin type."[6] These are complex substances composed of polysaccharide, lipid and protein, and have been obtained from a variety of the Gram-negative enteric bacilli. They are of utmost immunological importance in such organisms, since they comprise the essential immunizing antigen and also reveal the toxic properties commonly associated with these bacteria. Choucroun[7,8] has reported the presence of a toxic fraction in paraffin oil extracts of tubercle bacilli which apparently induces acquired resistance. This is a polysaccharide ester of mycolic acid, and may be related to the Boivin type of antigen.

Since Boivin's original demonstration of the extractability of antigenic complexes by trichloracetic acid,[6] several other methods have been described for accomplishing this end. Because we had no foreknowledge of the relative susceptibility of the tubercle bacillus to these extraction methods, we decided to employ them all on a comparative basis. The procedure of Raistrick and Topley[9] makes use of trypsin digestion; Morgan[10] employed diethylene glycol, and Walker[11] used urea for this purpose.

Preparation of Complexes. Human $H_{37}Rv$ bacilli were cultured on a modified Long-Seibert synthetic medium. At the end of 3 weeks the organisms were separated from the medium and washed with distilled water. A portion of these bacilli was washed in 50%, and then in absolute acetone in a refrigerated centrifuge. Immediately afterward the bacilli were dried in a vacuum dessicator over sulphuric acid. The remainder were employed in the fresh moist state. This difference in treatment was instigated by uncertainty as to the effect of even a short period of acetone treatment upon the organisms in view of the presence of acetone soluble fats in tubercle bacilli. Batches of the cells were extracted as shown in Table I, where the

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[4] Unpublished observations.

[5] Raffel, S., *Am. Rev. Tuberc.*, 1946, **54**, 564.

[6] Boivin, A., Mesrobeanu, I., Mesrobeanu, L., and Nestorescu, B., *C. R. Soc. Biol.*, 1934, **115**, 306.

[7] Choucroun, N., *Science*, 1943, **98**, 327.

[8] Choucroun, N., *Science*, 1947, **105**, 46.

[9] Raistrick, H., and Topley, W. W. C., *Brit. J. Exp. Path.*, 1934, **15**, 113.

[10] Morgan, W. T. J., *Biochem. J.*, 1937, **31**, 2003.

[11] Walker, J., *Biochem. J.*, 1940, **34**, 325.

TABLE I.
Essential Information Concerning the Extraction of Complexes from Tubercle Bacilli.

Bacillary batch No.	Treatment prior to extraction	Grams of bacilli extracted	Extraction method	Mg of lyophilized complex obtained
1	Washed with water, acetone dried	15.6 (dry wt)	Boivin—trichloracetic acid	219.0
2	Washed with water	10 (moist wt)	” ”	50.0
3	Washed with water, acetone dried	15.9 (dry wt)	Raistrick and Topley—trypsin digestion	257.0
4	Washed with water	25.2 (moist wt)	Walker—urea	51.4
5	Washed with water, acetone dried	15.4 (dry wt)	Morgan—diethylene glycol	26.5

yields of the individual complexes are also indicated. All of the complexes were lyophilized and stored in a carbon dioxide box. They formed colloidal suspensions when dissolved in saline or water.

During the course of their preparation these substances were subjected to the following physical manipulations calculated to free the extracts of intact bacilli:

The 2 trichloracetic acid preparations were freed of most bacteria by centrifugation in the cold. The supernatants were then ultracentrifuged for 15 minutes at 12,000 r.p.m. Following this the extracts were passed through Jena 5/3 glass filters, and were then lyophilized. The trypsin and urea preparations were freed of most of the organisms by Sharples centrifugation. These fluids were filtered through Chamberland L_3 candles prior to lyophilization.

The diethylene glycol extract, because of its smaller volume and the nature of the fluid, was spun in the ultracentrifuge at 25,000 r.p.m. for one hour. This material was subsequently filtered through a Chamberland L_3 candle also.

Treatment of guinea pigs. These lyophilized preparations were freshly dissolved for each immunizing injection. Fifty-three guinea pigs weighing between 400 and 500 g, and negative in skin test to Old Tuberculin 1:10, were given the first injections of these complexes on August 19, 1946. Ten or 11 animals were apportioned to each of the preparations. These groups of guinea pigs will be referred to by the numbers used to designate the bacillary batches in Table I. The dose of each complex administered was 0.5 mg in 0.25 cc of saline, injected subcutaneously. Three weeks later skin tests with O.T. 1:10 proved negative in all groups. One month following the first injection, second injections of the same dose of the respective complexes were administered, this time intracutaneously. This was repeated a month later, subcutaneously. Thus, during a period of 8 weeks these animals received 3 doses of complex, totaling 1.5 mg for each animal.

Twelve weeks after the first injection of each complex, routine skin tests were carried out with undiluted O.T. and the results were quite unexpected. Every one of the 43 animals comprising Groups 1, 2, 3 and 4 developed exceedingly large necrotizing reactions, averaging 29 mm in diameter. These were unusually severe in that practically the entire areas were necrotic within 24 hours. One week later tests were repeated in 12 of these guinea pigs with less concentrated O.T. (1:10). The average diameter of reaction was 25 mm, and in 10 of these necrosis occurred. None of the animals receiving the diethylene glycol complex developed sensitivity to O.T., either at this time nor during several succeeding months of injections.

One of the animals of Group 4 (urea complex) which died as a result of the initial tuberculin reaction, showed marked tuberculosis involving the axillary lymph nodes and the spleen. Histologic examination revealed lesions with bacilli in these locations. All of the remaining animals in Groups 1,

TABLE II.
Gross Tuberculosis in Regional Lymph Nodes, Abdominal and Thoracic Viscera of Guinea Pigs Receiving Injections of Complexes.

Guinea pig group	No. of pigs in groups	No. showing involvement of lymph nodes regional to inject. (axillary and ing.)	No. with involvement of liver and/or spleen	No. with involvement of lungs and/or tracheobronchial lymph nodes
Trichlor. extract of dried bacilli	9	8	9	7
Trichlor. extr. of fresh bacilli	9	8	8	6
Trypsin extr. dried bacilli	11	8	6	4
Urea extr. fresh bacilli	8	7	7	5
Total	37	31	30	22

2, 3 and 4 were subsequently examined at autopsy for the degree and extent of dissemination of tuberculosis. The overall results are shown in Table II. Thirty-one of the 37 animals showed gross evidence of infection at 3 months or later following the first injection of the respective complexes. The animals which failed to reveal such evidence had previously responded to Old Tuberculin with skin reactions equal in severity to those seen in the pigs with obvious disease. Of the 31 animals, most showed advanced infection, and a few were striking in this regard. As indicated in Table II infection was most evident in the abdominal viscera and in the axillary and inguinal lymph nodes draining the areas of injections of the complexes, while a smaller proportion of animals showed disease also in the lungs or tracheobronchial lymph nodes. In no case was there infection in the lungs or tracheobronchial nodes in the absence of abdominal visceral or axillary-inguinal node involvement. It is thus rather apparent that infection was consequent to the injections, and was not spontaneous as the result of aspiration of bacilli from the environment. Added to this evidence is the fact that groups of normal unused guinea pigs as well as those receiving other, noninfectious vaccinating treatments such as the diethylene glycol complex and various preparations of proteins derived from the tubercle bacillus, were housed in the same large room during the entire period of this experiment. These have never responded to repeated tests with concentrated Old Tuberculin, nor has evidence of tuberculosis been encountered in autopsies of many such animals. Furthermore, it has been a general experience as well as our own that guinea pigs are very unliable to spontaneous infection with *M. tuberculosis* even after intimate and prolonged contacts.

Since now it was apparent that the complexes which had been intended as immunizing agents were actually infectious, we undertook to determine what numbers of bacilli could have escaped the centrifugation and filtration procedures employed in the preparation of these substances. For this purpose the total residual lyophilized complexes were dissolved in saline and ultracentrifuged at 25,000 r.p.m. for one hour. No visible sediments were thrown down, but the bottoms of the tubes were carefully scraped and smears made of the scrapings. These were examined for acid-fast bacilli. As shown in Table III, very few tubercle bacilli were encountered, although in every case the sediments represented amounts of complex greatly in excess of that received by the animals.

TABLE III.
Results of Search for Tubercle Bacilli in the Ultracentrifugal Sediments of Complexes.

Complex	Mg of complex ultracentrifuged	Result of examination of total recoverable sediment
1. Trichloracetic acid extr. of dried bacilli	200	6 bacilli
2. Trichloracetic acid extr. fresh bacilli	35	2 possible bacilli, not distinct
3. Trypsin digest extr. dried bacilli	240	4 bacilli
4. Urea extr. fresh bacilli	35	8 ”

It was now evident that these guinea pigs had developed a surprising incidence and extent of infection as the result of the very small numbers of tubercle bacilli which they had received. Since we had made frequent use of this strain of organisms ($H_{37}Rv$) for infecting guinea pigs in the past, we were able to gather from previous records the relative degrees of tuberculosis established at various comparable intervals following infection with measured doses of bacteria. Guinea pigs which had received 2 ranges of infective doses by the subcutaneous route were reviewed for this purpose. The first group had been infected with 0.75 or 1.0 mg (moist weight) of bacilli, and the second had received doses of 0.05, 0.075 or 0.10 mg. A third group of pigs was available for a much more direct and striking comparison. These animals had received acetone-dried bacilli of the same batch employed for the preparation of the trichloracetic extract complex (No. 1, Table I) and the trypsin complex (No. 3, Table I). These bacilli failed to grow on glycerine egg medium during an 8-week period of observation. The organisms were administered to 13 guinea pigs in doses of 10 mg dry weight for each subcutaneous injection. A total of 40 mg was given to each animal in 4 injections during a period of 3 months.

In order to compare these 3 groups with the animals receiving complexes, the Feldman index[12] was employed to score the gross tuberculosis observed at autopsy. This index takes account of the extent of involvement of the lymph nodes draining the region of inoculation, the spleen, liver, lungs and the tracheobronchial lymph nodes.

The average indices for each group of animals are portrayed in Table IV and Fig. 1. It may be noted that the averages of organ involvement in the animals receiving complexes were uniformly greater at the various examination intervals than those observed in the guinea pigs injected with massive doses of acetone dried bacilli, and were not far below those of the animals infected with the 0.05 to 0.10 mg doses of fresh young culture organisms. The guinea pigs which had received 0.75 to 1.0 mg amounts of bacilli to induce infection showed more definitely advanced disease at comparable intervals following infection.

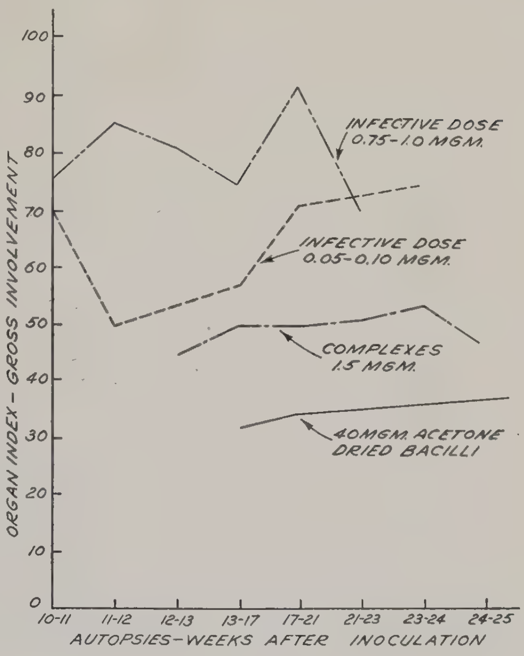

Fig. 1.

Discussion. The factors which should enter into consideration of these results are the following:

(a) The animals of the "complex groups" received a total of 1.5 mg of material of which much greater amounts revealed sparse tubercle bacilli in ultracentrifugal sediments. In contrast, the groups of guinea pigs included in the table for purposes of comparison received relatively enormous numbers of bacteria of the same strain, and in the case of the acetone dried organisms, of the same batch and at the same time.

(b) The bacilli which remained in the complexes had been subjected to chemical and physical procedures as well as aging in the lyophilized state. The organisms employed for infecting 2 of the comparative groups of animals were from 2-week-old cultures, freshly suspended in saline immediately be-

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[12] Feldman, W. H., *Am. Rev. Tuberc.*, 1943, **48**, 248.

TABLE IV.
Scoring of Guinea Pig Organs for Extent of Grossly Observable Tuberculosis at Various Periods After Infection.

Wks after infection	Infected with: 0.75 or 1.0 mg fresh bacilli — No. of G pigs	0.75 or 1.0 mg fresh bacilli — Avg score	0.05-0.10 mg fresh bacilli — No. of G pigs	0.05-0.10 mg fresh bacilli — Avg score	40 mg acetone dried bacilli* — No. of G pigs	40 mg acetone dried bacilli* — Avg score	bacilli present in complexes† — No. of G pigs	bacilli present in complexes† — Avg score
10-11	23	76	2	70				
11-12	8	86	2	50				
12-13	7	81					1	45
13-17	12	74	7	58	2	32	9	50
17-21	2	92	8	71	8	34	2	50
21-23	1	70					4	51
23-24			1	75			10	53
24-25							11	47
26-32					3	38		

* Infection interval dated from 1st injection of 10.0 mg of bacilli. A total of 40 mg was eventually injected into all pigs.

† Infection interval dated from 1st injection of 0.50 mg of the various complexes. A total of 1.5 mg was eventually injected into all pigs.

fore use. The acetone-dried bacilli given to the third comparative group had been subjected to considerably less manipulation than was accorded those organisms remaining in the complexes.

(c) The infection intervals in the "complex animals" are dated from the time of first injection of these substances. The 3 injections actually spanned a period of 8 weeks, and with the small numbers of organisms involved, some of the animals may well have received no bacilli on the first, and perhaps the second, injection. The intervals after infections set forth in Table II may therefore be considerably exaggerated in some of these animals.

It appears then that the ability of the very sparse numbers of tubercle bacilli present in the complexes to produce a degree of infection greater than that found following massive doses of similarly treated organisms alone must depend upon some function of the complexes themselves. So far as can be deduced from these experiments, the complexes acted in the capacity of a virulence factor, permitting small numbers of possibly "injured" bacilli to establish themselves in the normal host and to produce progressive infection.

Whether such complexes can function in the same capacity in relation to attenuated bacilli, such as B.C.G., is being investigated at the present time. The possible role of these substances in inducing acquired resistance is also under study.

Conclusions. The Boivin type of antigenic complexes derived from the human strain ($H_{37}Rv$) of *M. tuberculosis* appear to abet generalized infection in guinea pigs receiving very small numbers of organisms. It is suggested that such complexes may function as a "virulence factor" for the tubercle bacillus in a susceptible species of host. The possible relationship of such a factor to acquired resistance to the tubercle bacillus is under investigation.

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